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THE HARVEY LECTURES

THE HARVEY LECTURES

DELIVERED UNDER THE AUSPICES OF

The HARVEY SOCIETY of NEW YORK

1944-1945

UNDER THE PATRONAGE OF THE NEW YORK
ACADEMY OF MEDICINE

BY

DR. E. NEWTON HARVEY
DR. GEORGE WELLS BEADLE
DR. SELMAN A. WAKSMAN
DR. EDWIN B. ASTWOOD
DR. JEAN OLIVER
DR. WILLIAM F. WINDLE
DR. HERMANN OTTO LAURENZ FISCHER
DR. FRANCIS O. SCHMITT

SERIES XL

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THE HARVEY SOCIETY

A SOCIETY FOR THE DIFFUSION OF KNOWLEDGE
OF THE MEDICAL SCIENCES

CONSTITUTION

I

This Society shall be named the Harvey Society.

II

The object of this Society shall be the diffusion of scientific knowledge in selected chapters in anatomy, physiology, pathology, bacteriology, pharmacology, and physiological and pathological chemistry, through the medium of public lectures by men who are workers in the subjects presented.

III

The members of the Society shall constitute three classes: Active, Associate, and Honorary members. Active members shall be laboratory workers in the medical or biological sciences, residing in the City of New York, who have personally contributed to the advancement of these sciences. Associate members shall be meritorious physicans who are in sympathy with the objects of the Society, residing in the City of New York. Members who leave New York to reside elsewhere may retain their membership. Honorary members shall be those who have delivered lectures before the Society and who are neither Active nor Associate members. Associate and Honorary members shall not be eligible to office, nor shall they be entitled to a vote.

Members shall be elected by ballot. They shall be nominated to the Executive Committee and the names of the nominees shall accompany the notice of the meeting at which the vote for their election will be taken.

IV

The management of the Society shall be vested in an Executive Committee to consist of a President, a Vice-President, a Secretary, a Treasurer, and three other members, these officers to be elected by ballot at each annual meeting of the Society to serve one year.

V

The Annual Meeting of the Society shall be held at a stated date in January of each year at a time and place to be determined by the Executive Committee. Special meetings may be held at such times and places as the Executive Committee may determine. At all meetings ten members shall constitute a quorum.

VI

Changes in the Constitution may be made at any meeting of the Society by a majority vote of those present after previous notification to the members in writing.

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OFFICERS

1945–1946

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VINCENT DU VIGNEAUD, *Vice-President*
COLIN M. MACLEOD, *Treasurer*
EDGAR G. MILLER, JR., *Secretary*

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1945–1946

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FRED W. STEWART
PHILIP D. MCMASTER

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Secretary: GEORGE B. WALLACE

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S. J. MELTZER
EDWARD K. DUNHAM

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President: GRAHAM LUSK
Vice-President: SIMON FLEXNER
Treasurer: FREDERIC S. LEE
Secretary: GEORGE B. WALLACE

Council:
C. A. HERTER
S. J. MELTZER
JAMES EWING

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Vice-President: JAMES EWING
Treasurer: EDWARD K. DUNHAM
Secretary: GEORGE B. WALLACE

Council:
SIMON FLEXNER
THEO. C. JANEWAY
PHILIP H. HISS, JR.

1908–1909

President: JAMES EWING
Vice-President: SIMON FLEXNER
Treasurer: EDWARD K. DUNHAM
Secretary: FRANCIS C. WOOD

Council:
GRAHAM LUSK
S. J. MELTZER
ADOLF MEYER

1909–1910*

President: JAMES EWING
Vice-President: THEO. C. JANEWAY
Treasurer: EDWARD K. DUNHAM
Secretary: FRANCIS C. WOOD

Council:
GRAHAM LUSK
S. J. MELTZER
W. J. GIES

* At the Annual Meeting of May 18, 1909, these officers were elected. In publishing the 1909–1910 volume their names were omitted, possibly because in that volume the custom of publishing the names of the incumbents of the current year was changed to publishing the names of the officers selected for the ensuing year.

1910–1911

President: Simon Flexner
Vice-President: John Howland
Treasurer: Edward K. Dunham
Secretary: Haven Emerson

Council:
Graham Lusk
S. J. Meltzer
James Ewing

1911–1912

President: S. J. Meltzer
Vice-President: Frederic S. Lee
Treasurer: Edward K. Dunham
Secretary: Haven Emerson

Council:
Graham Lusk
James Ewing
Simon Flexner

1912–1913

President: Frederic S. Lee
Vice-President: Wm. H. Park
Treasurer: Edward K. Dunham
Secretary: Haven Emerson

Council:
Graham Lusk
S. J. Meltzer
Wm. G. MacCallum

1913–1914

President: Frederic S. Lee
Vice-President: Wm. G. MacCallum
Treasurer: Edward K. Dunham
Secretary: Augustus B. Wadsworth

Council:
Graham Lusk
Wm. H. Park
George B. Wallace

1914–1915

President: Wm. G. MacCallum
Vice-President: Rufus I. Cole
Treasurer: Edward K. Dunham
Secretary: John A. Mandel

Council:
Graham Lusk
Frederic S. Lee
W. T. Longcope

1915–1916

President: George B. Wallace*
Treasurer: Edward K. Dunham
Secretary: Robert A. Lambert

Council:
Graham Lusk
Rufus I. Cole
Nellis B. Foster

* Dr. William G. MacCallum resigned after election. On Doctor Lusk's motion Doctor George B. Wallace was made President—no Vice-President was appointed.

1916–1917

President: George B. Wallace
Vice-President: Rufus I. Cole
Treasurer: Edward K. Dunham
Secretary: Robert A. Lambert

Council:
Graham Lusk*
W. T. Longcope
S. R. Benedict
Hans Zinsser

1917–1918

President: Edward K. Dunham
Vice-President: Rufus I. Cole
Treasurer: F. H. Pike
Secretary: A. M. Pappenheimer

Council:
Graham Lusk
George B. Wallace
Frederic S. Lee
Peyton Rous

1918–1919

President: Graham Lusk
Vice-President: Rufus I. Cole
Treasurer: F. H. Pike
Secretary: K. M. Vogel

Council:
Graham Lusk
James W. Jobling
Frederic S. Lee
John Auer

1919–1920

President: Warfield T. Longcope
Vice-President: S. R. Benedict
Treasurer: F. H. Pike
Secretary: K. M. Vogel

Council:
Graham Lusk
Hans Zinsser
Frederic S. Lee
George B. Wallace

1920–1921†

President: Warfield T. Longcope
Vice-President: S. R. Benedict
Treasurer: A. M. Pappenheimer
Secretary: Homer F. Swift

Council:
Graham Lusk
Frederic S. Lee
Hans Zinsser
George B. Wallace

* Doctor Lusk was made Honorary permanent Counsellor.

† These officers were elected at the Annual Meeting of May 21, 1920, but were omitted in the publication of the 1919–1920 volume.

1921–1922

President: Rufus I. Cole
Vice-President: Stanley R. Benedict
Treasurer: A. M. Pappenheimer
Secretary: Homer F. Swift

Council:
Graham Lusk
Hans Zinsser
H. C. Jackson
W. T. Longcope

1922–1923

President: Rufus I. Cole
Vice-President: Hans Zinsser
Treasurer: Charles C. Lieb
Secretary: Homer F. Swift

Council:
Graham Lusk
W. T. Longcope
H. C. Jackson
S. R. Benedict

1923–1924

President: Eugene F. DuBois
Vice-President: Homer F. Swift
Treasurer: Charles C. Lieb
Secretary: George M. Mackenzie

Council:
Graham Lusk
Alphonse R. Dochez
David Marine
Peyton Rous

1924–1925

President: Eugene F. DuBois
Vice-President: Peyton Rous
Treasurer: Charles C. Lieb
Secretary: George M. Mackenzie

Council:
Graham Lusk
Rufus I. Cole
Haven Emerson
Wm. H. Park

1925–1926

President: Homer F. Swift
Vice-President: H. B. Williams
Treasurer: Haven Emerson
Secretary: George M. Mackenzie

Council:
Graham Lusk
Eugene F. DuBois
Walter W. Palmer
H. D. Senior

1926–1927

President: Walter W. Palmer
Vice-President: Wm. H. Park
Treasurer: Haven Emerson
Secretary: George M. Mackenzie

Council:
Graham Lusk
Homer F. Swift
A. R. Dochez
Robert Chambers

1927–1928

President: Donald D. Van Slyke
Vice-President: James W. Jobling
Treasurer: Haven Emerson
Secretary: Carl A. L. Binger

Council:
Graham Lusk
Russell L. Cecil
Ward J. MacNeal
David Marine

1928–1929

President: Peyton Rous
Vice-President: Horatio B. Williams
Treasurer: Haven Emerson
Secretary: Philip D. McMaster

Council:
Graham Lusk
Robert Chambers
Alfred F. Hess
H. D. Senior

1929–1930

President: G. Canby Robinson
Vice-President: Alfred F. Hess
Treasurer: Haven Emerson
Secretary: Dayton J. Edwards

Council:
Graham Lusk
Alfred E. Cohn
A. M. Pappenheimer
H. D. Senior

1930–1931

President: Alfred E. Cohn
Vice-President: J. G. Hopkins
Treasurer: Haven Emerson
Secretary: Dayton J. Edwards

Council:
Graham Lusk
O. T. Avery
A. M. Pappenheimer
S. R. Detwiler

1931–1932

President: J. W. Jobling
Vice-President: Homer W. Smith
Treasurer: Haven Emerson
Secretary: Dayton J. Edwards

Council:
Graham Lusk
S. R. Detwiler
Thomas M. Rivers
Randolph West

1932–1933

President: Alfred F. Hess
Vice-President: Haven Emerson
Treasurer: T. M. Rivers
Secretary: Edgar Stillman

Council:
Graham Lusk
Hans T. Clark
Walter W. Palmer
Homer W. Smith

1933–1934

President: Alfred F. Hess
Vice-President: Robert K. Cannan
Treasurer: Thomas M. Rivers
Secretary: Edgar Stillman

Council:
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Robert F. Loeb
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Treasurer: Thomas M. Rivers
Secretary: Randolph H. West

Council:
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B. S. Oppenheimer
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President: Robert K. Cannan
Vice-President: Eugene L. Opie
Treasurer: Thomas M. Rivers
Secretary: Randolph H. West

Council:
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Homer W. Smith
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1936–1937

President: Eugene L. Opie
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Treasurer: Thomas M. Rivers
Secretary: McKeen Cattell

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Council:
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Treasurer: KENNETH GOODNER
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Treasurer: KENNETH GOODNER
Secretary: THOMAS FRANCIS, JR.

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DECOMPRESSION SICKNESS AND BUBBLE FORMATION IN BLOOD AND TISSUES[1,2]

E. NEWTON HARVEY

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INTRODUCTION

AFTER the last World War, 1914–1918, an editorial in the *Journal of the American Medical Association,* in referring to medical aspects of aviation and praising the work that had been done to protect aviators against the effects of altitude, spoke of the necessity of speed and the ability to climb above an opponent. At that time bombing and observation planes flew "for hours at altitudes of 12,000 to 15,000 feet" while pursuit planes attained "the enormous altitude of 18,000 and 20,000 feet" (1). In the second World War a service ceiling of well over 40,000 feet has been attained, a height where even the breathing of pure oxygen is not sufficient to supply the needs of the body. Without the protection of a pressurized cabin, man has become the limiting factor to high ascent.

But considerably below 40,000 feet, a series of symptoms have been described which cannot be attributed to any one of the three common and obvious difficulties at great height: (1) lack of oxygen; (2) ear and sinus trouble, due to non-equalization of pressure between middle ear (or sinuses) and the external atmosphere; (3) intestinal pains, due to the expansion of gas in the digestive tract. A fourth group of additional symptoms may appear and affect various parts of the body. These range from a mild rash or ache to unbearable pain near the joints, from tightness in the chest to serious coughing spells and from hyperesthesias or anesthesias to convulsions, paralysis and syncope.

[1] Lecture delivered October 26, 1944.

[2] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Princeton University.

The individual is definitely incapacitated, sometimes seriously. Since the symptoms may begin to appear as low as 25,000 feet and are marked at 35,000 feet, they limit the altitude at which an open plane can be flown, for these heights are well below the point (38,000 ft.) at which pure oxygen just begins to fail in supplying the retina and brain. Any of the above group of symptoms fully described in Armstrong's (2) classic book on Aviation Medicine, constitute decompression sickness,[3] since they result from decompressing the body from one atmosphere to lower values, an analogous change to the return of a person to one atmosphere after a previous exposure to compressed air. Decompression sickness may also be applied to the symptoms resulting from the latter change, although the older terms, compressed air illness or caisson or diver's disease, have been more frequently used.

In both compression-decompression experiments and in low pressure or "altitude" experiments, excess gas is dissolved in the body. The tissues are saturated at a certain gas pressure, producing a similar tension of dissolved gas in the cells and tissues, and then the gas pressure is decreased. The procedure can be compared to removing the cap from a soda water bottle or exhausting a tube of water with an air pump. Under certain conditions bubbles will form in both cases. It has long been recognized and appears quite certain that compressed air illness is due to bubble formation in blood and tissues. By analogy the symptoms at high altitude might also be expected to result from bubbles, although the question, until recently, had by no means been settled and other theories had been proposed. Indeed, the formation of bubbles in animals at low barometric pressures had been denied by the father of barometric studies, Paul Bert.

Early in the organization of the Committee on Medical Research, the division of Aviation Medicine foresaw the seriousness of decompression symptoms and established the Subcommittee on Decompression Sickness, with Dr. John F. Fulton, of Yale Uni-

[3] Armstrong (2) has suggested the word aeroembolism, but this term implies unproven ideas about the origin of the symptoms.

versity, as able Chairman. The present basic study was undertaken in order to separate and analyze the fundamental factors involved in the formation of bubbles, with special reference to the conditions found in animals, and with the purpose of applying these findings to the aviator. Critical terminal experiments can be carried out with animals that cannot be undertaken with man. Moreover, the whole problem of bubble formation in liquids was in a little known state at the time this work was started in May, 1942.

Many other groups of investigators have been sponsored by this Subcommittee. It is not possible, for security reasons, to refer to the vast amount of detailed data collected by these groups. The reader is referred to reports that will eventually be published, prepared under the direction of the responsible investigators—Behnke, Bronk, Blankenhorn and Ferris, Evans, Fulton, Greeley, Harvey, Hitchcock, Ivy, Jacobs, Knisley, Lawrence and Hamilton, Scott, Swindle, Whitaker, Blinks and Twitty—as well as the many Army and Navy experimental and training stations in this country, in Canada and in England.

It is a pleasure to express here my sincere appreciation of the keen interest, fertile ideas and extended experimentation of the men who have collaborated on the Princeton project[4]—Dr. Wm. D. McElroy and Mr. A. H. Whiteley, members for the full period of the contract, and Dr. D. C. Pease, Dr. G. H. Warren, Dr. K. W. Cooper and Dr. Wm. Kleinberg, part of whose time has been devoted to the work. I am also particularly grateful to Professor Henry Eyring, of the Chemistry Department, Princeton University, for much helpful discussion, and especially to Mr. D. K. Barnes, who has worked out a detailed theory of the separation of a gas phase from a liquid and clarified our knowledge of the conditions for stability and growth of gas masses.

HISTORY

Probably the first bubble recorded in animals was that seen by Robert Boyle (3) in the eye of a snake which he had placed

[4] The results of this work will be published in a series of papers in the *Journal of Cellular and Comparative Physiology* for 1944.

under his newly invented air pump in 1670. Boyle's remarks are so pertinent to our subject and so prophetic of what has now been realized in aviation that I quote him in full. After describing an experiment with fresh blood and another with fresh milk which boiled vigorously on evacuation with his pneumatical engine, he says:

Note, that the two foregoing Experiments were made with an Eye cast upon the inquiry, that I thought might be made; Whether, and how far the destructive operation of our Engin upon the included Animal, might be imputed to this, that upon the withdrawing of the Air, besides the removal of what the Airs presence contributes to life, the little Bubbles generated upon the absence of the Air in the Blood, juices, and soft parts of the Body, may by their Vast number, and their conspiring distension, variously streighten in some places, and stretch in others, the Vessels, especially the smaller ones, that convey the Blood and Nourishment; and so by choaking up some passages, and vitiating the figure of others, disturb or hinder the due circulation of the Blood? Not to mention the pains that such distensions may cause in some Nerves, and membranous parts, which by irritating some of them into convulsions may hasten the death of Animals, and destroy them sooner by occasion of that irritation, than they would be destroyed by the bare absence or loss of what the Air is necessary to supply them with. And to shew, how this production of Bubbles reaches even to very minute parts of the Body, I shall add on this occasion (hoping that I have not prevented my self on any other) what may seem somewhat strange, what I once observed in a *Viper,* furiously tortured in our Exhausted Receiver, namely, that it had manifestly a conspicuous Bubble moving to and fro in the waterish humour of one of its Eyes.

Although sporadic observations were made after Boyle, real knowledge of the effects of varying atmospheric pressures began with Paul Bert's systematic study, published as *La pression barométrique* in 1878 (4). Bert demonstrated the presence of bubbles in the blood and tissues of animals after compressed air experiments but denied the existence of such bubbles in animals exposed to low pressures, although Hoppe (5) in 1857 had previously reported bubbles to be present in animals at 50 mm. Hg (62,000 ft.). To further study the problem, Hill and Greenwood (6) in 1910 exposed 8 mice, 2 guinea pigs, a cat, a kitten and a rabbit to 50 mm. Hg air pressure until the animals died and then examined them post-mortem. No bubbles were found in any except the rabbit whose heart and large vessels were full

of air. They concluded that in general Paul Bert was right. Actually, both Hoppe and Bert were correct for we now know that resting animals at high altitudes rarely develop bubbles but if they are exercised with vigorous muscle contraction, bubbles appear in the blood quite regularly. These conflicting results, as well as the doubts expressed by various workers that bubbles appear in blood at altitudes, must be attributed to the chance variation in amount of movement taking place in the animals under experimentation.

The bad effects of deep diving have long been known but the problem of illness after exposure to high air pressures became particularly important with the invention in 1840 of the caisson, a compressed air chamber for sinking shafts or tunnels or for building piers under water. With the extended use of this device and the compressed air diving suit in the latter part of the last century, diver's or caisson disease became common. A series of words were coined by the workers to describe the symptoms. Among these the "bends," the "chokes" (or "chokers"), the "itch" and the "staggers" are the most picturesque and describe quite adequately joint pains, respiratory difficulties, skin eruptions and paralyses. The experience from actual flying and in high altitude chambers indicates that all the above symptoms may also occur. Skin trouble and nervous affections are rather rare but the bends and chokes are frequent and these words have become a permanent part of the scientific vocabulary of the subject.

Interest of the British Admiralty in diving led J. S. Haldane (7) with Boycott and Damant (8, 9) as well as Leonard Hill (10) to their extensive research in 1906 to 1908 while in the United States, beginning in 1935, Behnke (11, 12) and associates, Shaw, Willmon and Yarborough and Shilling (12a), have extended our knowledge, making special studies of nitrogen elimination, fat content and the use of helium for unusually deep diving to eliminate the dangers of oxygen poisoning and nitrogen narcosis. In Germany the classic contribution of Heller, Mager and von Schrotter (13) compiles the facts on diving up to 1900.

The symptoms of caisson disease could be immediately alleviated and a life often saved by recompressing the patient and then decompressing more slowly, allowing time for excess gas to be removed from the body. Chambers for this purpose were first introduced by Sir Ernest Moir about 1890. The increased pressure contracts the bubbles and this fact proves that symptoms only appear when bubbles reach a certain size. Experience at simulated high altitudes likewise indicates that recompression to lower altitudes (even a change from 35,000 to 25,000 feet) will also instantly relieve bends and chokes but upon immediate reascent the pain will reappear in exactly the same place.

Such facts lead to the inescapable conclusion that decompression sickness results from the formation of gas bubbles in the body and the multiplicity of symptoms reflects the region in which the bubbles grow or lodge. The relief or treatment of decompression sickness is an accomplished procedure—immediate recompression. Prophylaxis remains to be considered. If bubble formation could be prevented in the body when its tissues are supersaturated, bends would cease to be a problem.

It is obvious that a fundamental study of the conditions under which gas will separate from solution is absolutely essential. In addition the composition of the gas which forms the bubbles must be known and every possible fact that might bear on the problem. Consequently, a study of bubble formation has been undertaken under the simplest conditions easily attainable, in water saturated with a single gas in a glass vessel. This study has been supplemented by investigations of blood *in vitro* and also in the body of narcotized animals, for it is in this fluid, i.e., in the blood vessels, that bubbles most frequently occur. The condition may be called pompholyhemia and we have used this symptom in our study of the cat as an index to evaluate similar procedures which might cause or prevent decompression sickness in man.

BUBBLE FORMATION FROM EXCESS GAS

Many important principles can be demonstrated with a bottle of soda water in which carbon dioxide is dissolved at a tension of

3 or 4 atmospheres. If the chilled bottle has remained upright and undisturbed for some time, no bubbles will form when the cap is removed but on tipping the bottle so that soda water flows over the dry glass surface bubbles immediately appear, and on carefully pouring into a dry tumbler abundant bubbles cling to the walls. There may frequently be observed a chain of bubbles arising from a point where a gas mass remains sticking to the glass. Sometimes bubbles also arise from a clearly visible dust particle in the bulk of the liquid. If the tumbler is greasy, the hydrophobic region will be outlined by abundant bubbles which persist until the soda water has lost its excess gas. In fact this "bubble test" will tell how thoroughly a tumbler has been washed.

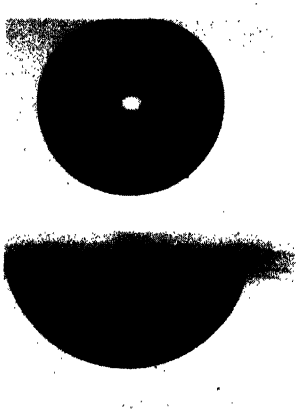
The bubbling and effervescence is all due to minute gas masses which stick to the dry walls or to dust particles and grow into bubbles as soon as the pressure release occurs. We can prove this by gently pouring the soda water into a scrupulously *clean* and *wet* tumbler, when no bubbles form, except during the first disturbance of the surface due to pouring. If some dry powder, such as infusorial earth, is now dropped into the quiet soda water the carbon dioxide separates with explosive violence but if the infusorial earth has been first boiled in water to remove its air films and then placed while wet into the soda water, not a single bubble will appear. However, a paraffined surface (hydrophobic), no matter how well cleaned, will always bubble profusely.

GAS NUCLEI

These effects are all due to small gas masses, or gas nuclei, which stick to any dirty, especially greasy, surface but not to clean wet glass. The sticking of gas is a matter of contact angles, which are well seen from the form of bubbles in capillary tubes or on surfaces. As shown in Figure 2, a bubble in a glass capillary filled with water has rounded (convex) ends—the contact angle (measured through the water) is zero; in a paraffined capillary the gas bubble has concave ends and the contact angle is about 108°. Since the form of gas bubbles in blood vessels is

similar to that of water in a glass tube, we may conclude that the endothelium is, on the average, hydrophilic, an important fact for the theory of bubble formation.

On a plane surface the form of bubbles is illustrated in Figure 1 and the contact angles are clearly visible. On a completely hydrophobic surface like glass, the zero contact angle means that no bubble can stick and no nucleus can remain but with any positive contact angle nuclei can stick and may be stable under certain conditions.



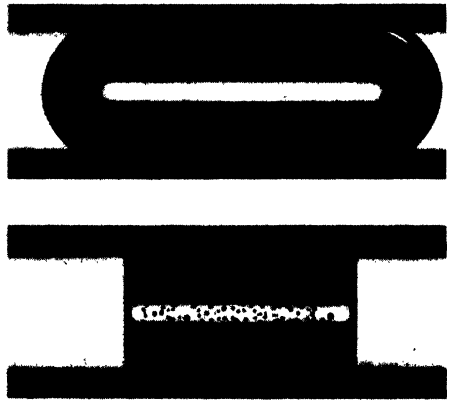


FIG. 1 (left). Photograph of an air bubble under a glass (above) and a paraffin surface (below).

FIG. 2 (right). Photograph of an air bubble in a glass capillary (above) and a hydrophobic capillary (below). In the latter the concave curvature of the air surface cannot be seen but it is indicated by the fact that the transmitted white band of light does not reach the edge of the gas cylinder. The air bubble on glass is 1.65 mm. across.

BUBBLE FORMATION FROM NEGATIVE PRESSURE

The development of bubbles in a liquid saturated with gas at one atmosphere and under one atmosphere pressure is uncommon but a number of examples are known. Such bubble formation is due to local decrease in hydrostatic pressure. A most interesting case was described by Osborne Reynolds (14) at a constric-

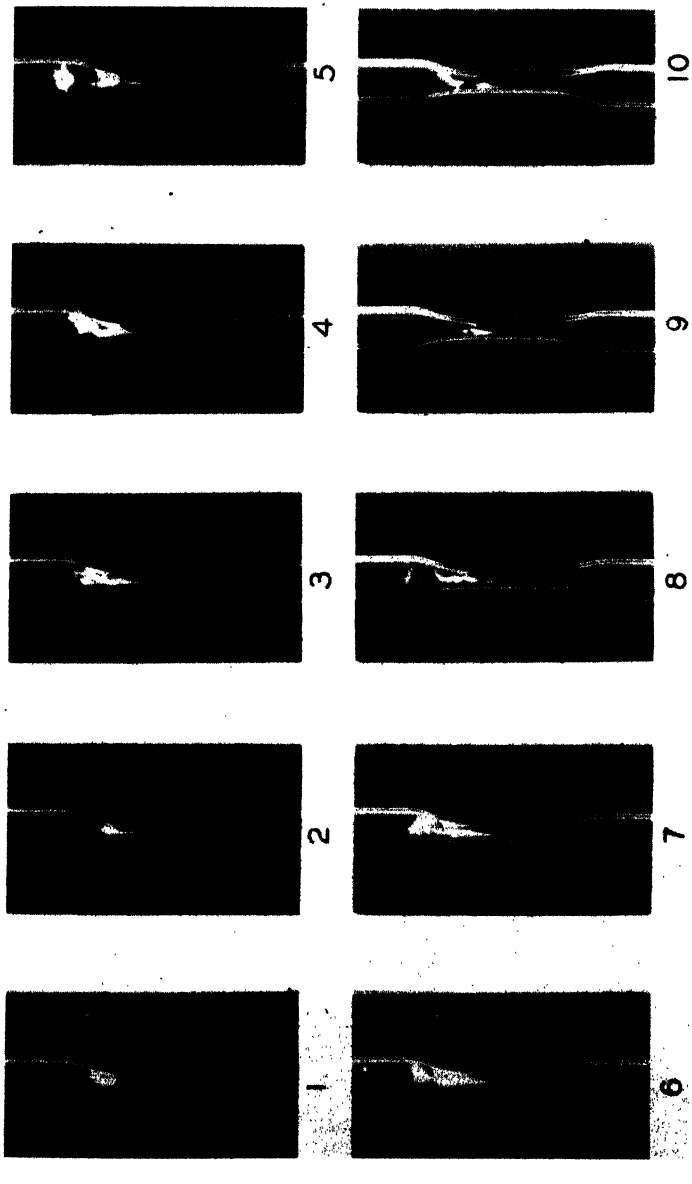


FIG. 3. Negative prints from a high-speed movie (1,680 frames per second) of cavitation in a Reynolds' tube of 10 mm. large diameter. Flow from bottom to top. Note the periodic cavitation (white in appearance) beginning at a particular spot on the narrow region of the tube. The individual frames are numbered.

tion in a pipe. When water flows through a tube which is narrowed at one place, the velocity is obviously increased at the constriction and, by Bernoulli's law, the pressure must decrease. In addition, violent turbulence and vortices appear.[5] If the constriction is narrow enough and the velocity sufficient, very low pressures will be produced and the water will suddenly break into cavities (cavitation) with a hissing sound. When the cavities collapse, which they do periodically, as shown in Figure 3, bubbles of gas remain and can be seen in the water leaving the constriction. Reynolds called the phenomenon "the boiling of water in an open tube at ordinary temperature." Whenever a local constriction occurs in a blood vessel, whether from a spasm or from passive squeezing between muscles, the pressure must be lowered at the constricted point, but it is doubtful if the velocity of flow is sufficient to produce Reynolds' cavitation in the body.

Another example is the cavitation which appears in water due to the passage of intense sound waves, or the bubbles which will form in a test tube of water at one atmosphere pressure if the bottom is hit a series of blows. Just as sound waves are made up of pressure changes, with increased followed by decreased components, so the series of blows involve increased followed by decreased pressure. A blow (or even vibration) to the human body at altitude would tend to bubble formation, but might not necessarily actually produce bubbles unless intense.

Whenever tensions are built up, as when a glass rod immersed in water is suddenly withdrawn, or when a propeller blade by rotation continually pulls away from the water, cavities may be formed and bubbles appear.

If the liquid, already supersaturated with gas, is subjected to a blow or any other treatment described above, the formation of bubbles is greatly facilitated. The results of cavitation become particularly important at high altitudes where the pressure is already near the vapor pressure of water, much less in compression-decompression experiments where the final pressure is one

[5] Dean (14a) believes most bubble formation in water free of gas nuclei is due to vortices.

atmosphere, far from the vapor pressure of water. A series of photographs of cavity and bubble formation in a tube of water at 110 mm. Hg pressure (the equivalent of 45,000 feet) after striking the bottom with a wooden block is reproduced in Figure 4.

The tensile strength of water is believed to be very high. This is demonstrated by experiments in which a slow steady pull is applied, as in those of Berthelot (15) and of Dixon (16), where clean degassed glass tubes were completely filled with water at a high temperature and the temperature then lowered, allowing the greater differential volume contraction of water as compared to the glass vessel to develop the pull. Negative pressures (tensions) as high as 150 atmospheres were found before the liquid suddenly broke, leaving a cavity.

This experiment is quite comparable to taking a piece of paper by two opposite edges and trying directly to pull it into two halves. The necessary effort is very great. On the other hand, by grasping one edge of a piece of paper with the two hands and twisting in opposite directions, the paper can be torn in two with the greatest of ease. The same amount of energy is required in either of the two methods but the rate of application of the energy (the power) necessary by the first is enormous, by the second very small.

Since tension is the negative equivalent of pressure[6] (i.e., negative force per unit area), if the area is small, enormous tensions may develop. In a liquid pressures or tensions quickly become equally distributed, as illustrated by Pascal's law, but, if the tension develops in a very short time interval, inertia and viscosity of the liquid may result in local high tensions (perhaps 100–1,000 atmospheres) so that the liquid is literally torn apart.

Considerable space has been devoted to this conception since we deal in the animal body with surfaces that *can* be *torn* apart and local pulls are involved rather than the uniform conditions of the Berthelot and Dixon experiments. Working with pre-

[6] Pressure in a gas can never become negative, but in a liquid, gel or solid high negative pressures develop if the material is subject to a pull.

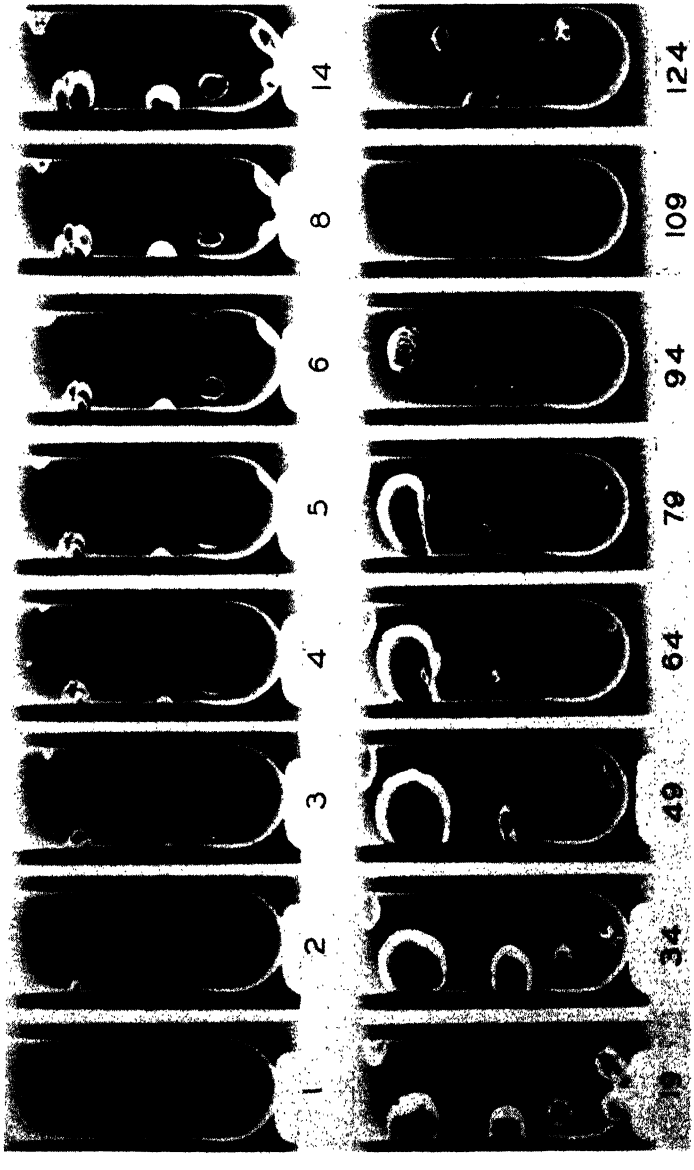


Fig. 4. Negative prints from a high-speed movie (1,200 frames per second) film of cavity formation in a glass tube (19.3 mm. diam.) filled with air saturated water at a pressure of 25 mm. Hg. when the bottom is struck a blow. The initial cavity oscillates in volume several times before the final bubbles start rising to the surface. The individual frames are numbered.

sumably gas nucleus free apparatus we can sometimes experimentally demonstrate the formation of cavities on the end of a glass rod drawn out of water in a narrow tube when the velocity reaches about 16 meters per sec. With corn syrup the velocity can be very low because of the inability of the viscid liquid to move into the space left by the rod, and with gelatin gel only a slight movement is sufficient to form a cavity. Moreover, the weight necessary to pull a glass rod out of gelatin is only a few kilograms and the local regions of high tension can be seen as strain patterns when viewed between crossed polaroids. Gas always moves into these cavities with extraordinary rapidity and a bubble is left when the cavities collapse.

That such a mechanism must be involved in the formation of bubbles in the animal can be demonstrated in excised tissues manipulated by pulling and cutting under conditions properly controlled to insure that extraneous gas nuclei are not introduced. Connective tissue is particularly prone to form bubbles by this procedure and it is perhaps significant that the bends are associated with regions rich in connective tissue.

THE PRESSURE DIFFERENCE, ΔP

From the preceding discussion it is apparent that the tendency for bubbles to form will depend on two factors, both on gas dissolved and also on pressure in the liquid. The difference between these may be called the pressure difference, ΔP. It is numerically equal to the gas tension, t, minus the hydrostatic pressure, P. The gas tension is determined by Henry's law, which states that at equilibrium the tension of gas dissolved in a liquid is proportional to the partial pressure, p, of gas which may be measured in atmospheres, in contact with the liquid. The hydrostatic pressure can be either positive or negative and may also be measured in atmospheres. The pressure difference, $\Delta P = t - P$, is one of the primary reasons for bubble formation in compression-decompression and in low pressure experiments.

If a gas nucleus is present, any change in ΔP will change its volume. However, if gas nuclei are absent, the question arises as

to how great ΔP must be before bubbles will form spontaneously, i.e., how much supersaturation is possible before *gas bubbles* appear *de novo* in a homogenous liquid at rest. A treatment based on thermodynamics and statistical mechanics indicates 100 to 1,000 atmospheres for spontaneous bubble formation. This is confirmed experimentally by Kenrick, Wismer and Wyatt (17), who have demonstrated that water, if undisturbed, may be brought into equilibrium with at least 150 atmospheres of gas without forming bubbles when the pressure is released.

This statement regarding high ΔP for spontaneous bubble formation applies also to water in contact with hydrophilic surfaces whether smooth or rough and to hydrophobic surfaces if molecularly smooth but, if pitted or containing cracks, gas nuclei can form *de novo* and grow to bubbles at less than 100–1,000 atmospheres gas tension. Theoretically, if a crack or a cone shaped cavity exists with a sufficiently small angle, *de novo nucleus formation* should occur without supersaturation with gas. This *de novo nucleus* may then grow to a bubble under the same conditions which control the growth of any other nucleus.

THE EFFECT OF DIFFERENT GASES—DIFFUSION

When compared at the same tension different gases will behave alike except when rates of diffusion are important. In this case not only ΔP but diffusion constants and especially solubility must be considered. A highly soluble gas like carbon dioxide, even at a low tension, may play an important rôle in the early growth of a bubble by diffusion because of the high concentration of carbon dioxide molecules.

The effect is well seen when a vapor cavity is produced in a liquid by negative hydrostatic pressure. If both carbon dioxide and nitrogen are dissolved at the same tension in the liquid surrounding the cavity, they will diffuse into the cavity at rates depending on their concentrations (since the diffusion constants are nearly alike) rather than their tensions. When the vapor cavity collapses there will be a much greater proportion of carbon dioxide than of nitrogen in the bubble that persists. Later the

proportion of carbon dioxide and nitrogen will adjust, so that at equilibrium an equal amount is present, i.e., a proportion that reflects their partial pressures.

Likewise, the size of bubbles will be greater if carbon dioxide rather than nitrogen is diffusing into them, both from the same tension. The rapid exit and entrance of carbon dioxide into bubbles is easily demonstrated by filling a long glass tube with layers of water alternately saturated with air and with carbon dioxide. The carbon dioxide layer may be colored with a dilute dye to render its boundaries visible. If minute bubbles are now produced in the air-saturated layer at bottom by striking the slightly evacuated tube a blow, the bubbles rise slowly, but when they pass into the carbon dioxide layer they jump in size, due to more rapid diffusion of carbon dioxide inward than of air out, and rise rapidly until they reach the air-saturated layer. Then carbon dioxide passes out of them rapidly; they suddenly shrink and rise slowly until the next carbon dioxide layer is encountered, when the process is repeated. We may imagine a rapid bubble growth in contracting muscle when carbon dioxide is produced, followed by the later replacement of this gas with nitrogen for permanent bubble formation.

STABILITY AND GROWTH OF GAS NUCLEI

Gas supersaturation is like salt supersaturation, with this exception. The presence of a minute crystal of the salt is sufficient to start crystallization of the whole salt solution, whereas a gas nucleus may enlarge to a certain point in a gas supersaturated liquid but will not grow indefinitely until certain critical conditions are surpassed. The conditions are simple and clear in a sphere. The surface tension of the gas-water interface exerts an inward pressure which is inversely proportional to the radius and amounts to an extra atmosphere in a bubble in pure water of 3μ radius. This excess pressure forces the gas into solution and the bubble disappears. Small spherical bubbles cannot exist unless there is excess gas in the liquid and the conditions for stability are that $\Delta P = 2\gamma/r$, when γ is the surface tension and r the radius of the bubble.

For a gas mass sticking to a surface the conditions of stability are much more complicated, since the configuration of the surface and the gas mass which may have two radii of curvature at

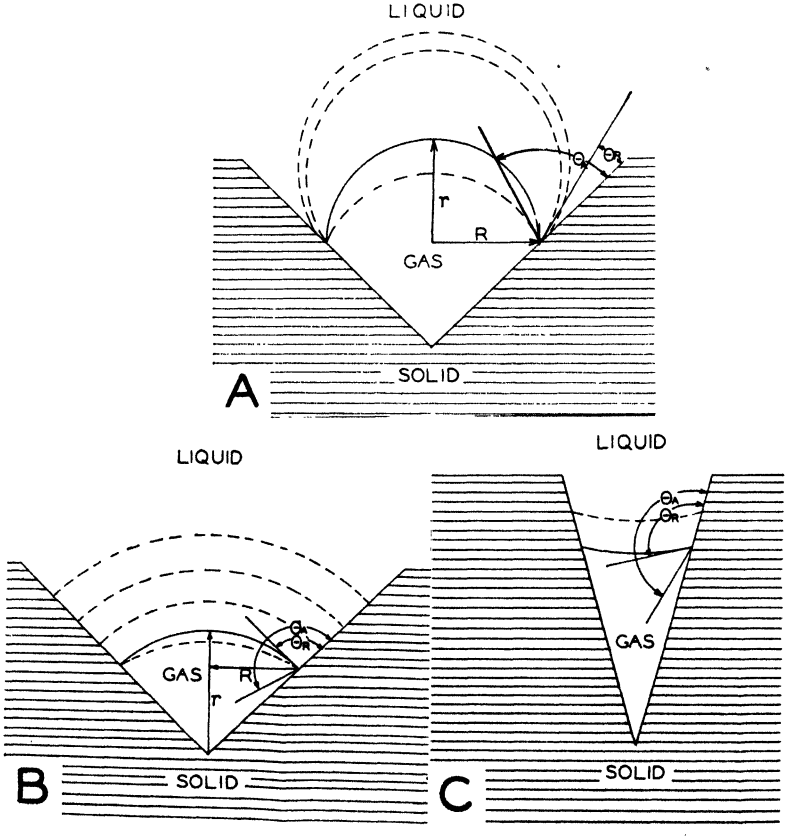


FIG. 5. A. A gas nucleus in a conical cavity of apical angle ψ, and small receding contact angle, θ_R. θ_A, advancing contact angle. R, radius of base; r, radius of curvature of gas-water interface at moment of instability (solid line). The dotted lines show stages of growth. The hemisphere limits stability. B. A similar gas nucleus in a conical cavity with large receding contact angle which limits stability. C. A gas nucleus in a cone of acute apical angle, ψ, so that $\theta_R > 90° + \psi/2$. Note curvature of gas-water surface.

right angles to each other, as well as the receding contact angle, which determines "creep" of the gas nucleus, are all important. It is not possible to describe in detail the conditions for stability of gas nuclei on surfaces but Figure 5 illustrates what will happen in cases which have been considered in a recent paper by Harvey *et al.* (18).

Wherever a surface is concave to the gas phase, as in Figure 5, C, the pressure in the gas is less than the gas tension in the water and gas would tend to move in from solution thereby continually enlarging the gas mass.

For every gas nucleus there is a certain ΔP below which it is stable and above which it grows by diffusion of gas. We may look upon a test tube of water, not especially cleaned to remove gas nuclei, as containing a population of gas nuclei of varying dimensions, each one of which will grow at a certain critical ΔP. If exhausted to one-half atmosphere, a group of bubbles will arise and then no more appear, but on reducing the pressure to one-quarter atmosphere another group of bubbles arises. This formation then ceases but at one-eighth atmosphere another group arises and so forth. At the vapor pressure, a ΔP of $760 - 20 = 740$ mm. Hg, the hydrostatic pressure, P, has been reduced as much as possible by evacuation but by striking the tube, a pressure pulse with a tension component is set up and P becomes less than the vapor pressure, giving rise to growth of a new series of gas nuclei with the formation of more bubbles. For convenience we can call all nuclei which will grow at or above the vapor pressure of water, gas macronuclei, while those which require a greater ΔP for continued growth are gas micronuclei. The distinction is quite arbitrary and merely represents a convenient line of demarcation.

REMOVAL OF GAS NUCLEI

For any experimental work with animals it is necessary to use liquids in containers or implements which have no gas nuclei sticking to them; otherwise quite erroneous conclusions may be drawn regarding the bubbling of blood, spinal fluid or tissues.

Any bubbles which appear in such an experiment may have come from gas nuclei on the surgical instrument or apparatus rather than from the blood or tissue [see Harvey *et al.* (19)].

The first necessity for avoidance of gas nuclei is to avoid hydrophobic material. This is not because a hydrophobic surface cannot be cleaned of gas nuclei, for it can, if always under water, but because the water runs off when exposed to air, and an air film then sticks by virtue of its contact angle. The surface should be so clean that a water film will cover it *completely* when removed to the air. If container and water are then well centrifuged, macronuclei or dust particles on glass can be removed and the water will not bubble at the vapor pressure. After treatment by this method, M HCl and M $NaHCO_3$ can be mixed without bubble formation and yeast can be grown in gas-nucleus-free culture medium with copious carbon dioxide production but without formation of bubbles. Filtering may also be employed to remove gas nuclei but it is essential to make certain that no gas masses are present on the filter, for filtering sometimes introduces more nuclei than it removes.

For removal of gas micronuclei two additional methods are available, both highly effective. One removes gas from solution by prolonged boiling or evacuation, thereby allowing the nucleus to disappear by solution in gas-free liquid; the other makes use of increased hydrostatic pressure, thereby forcing the nucleus into solution. The latter method is the most convenient, since the liquid retains its previous gas tension after a high pressure treatment and the difficult task of resaturation without introducing gas nuclei is avoided. We have placed both water and container in a steel chamber filled with water, and have used a hydrostatic pressure of 16,000 lb./in.^{2} (1,090 atmospheres) for 15 to 30 minutes. Such previously compressed water in a clean glass tube has remarkable properties. It can be heated to at least 202° C. before bursting into vapor, although evaporation from the surface is enormous. When intense high frequency sound waves are passed through, no cavitation occurs and no bubbles arise. Finally, if exhausted to the vapor pressure of water at 20° C., moderate knocks have no effect and only a very

severe blow, strong enough nearly to shatter the glass, will cause bubbles to form.

Although this water is gas-nucleus-free, bubbles can still be produced in it by procedures which increase ΔP to the 100 to 1,000 atmospheres necessary for spontaneous bubble formation—either by decreasing P, as when a glass rod is rapidly pulled out of the water with cavitation and bubble formation; or by increasing t, as when such water is electrolyzed and the gas concentration rapidly rises at the electrodes or when frozen and the formation of ice crystals (in which gas is insoluble) raises the gas concentration to a high value in pockets of the unfrozen liquid.

GAS NUCLEI IN BLOOD

With these fundamental principles in mind we may now turn to the animal and apply them to the observed facts. At altitude bubbles are rarely observed in resting animals but do appear in blood vessels as a result of muscle contraction, whereas in resting animals decompressed to one atmosphere from high air pressures the bubble formation is profuse in blood vessels, and many tissues are a froth of bubbles.

Since bubbles appear most readily in blood it was natural to test this liquid for gas nuclei. Previous experience with glass models and methods of removing gas nuclei have supplied the technique for drawing samples of blood into a long clean wet glass tube, or pompholygometer, one end of which is a cannula inserted and tied in the blood vessel. By appropriate clamps, stop-cocks and connections to a vacuum reservoir, successive samples of blood in the pompholygometer can be tested below the blood vapor pressure at 38° C. to see if gas nuclei are present which will grow into visible bubbles. The testing conditions of less than 47 mm. Hg are equivalent to an altitude of 63,000 feet (a ΔP of 713 mm.), far greater than the animal itself can stand.

The carotid blood when sampled in the above manner has been found to be free of gas nuclei in resting cats at ground level as well as in cats after a previous exposure to high altitude.[7] Even

[7] The word altitude used in this sense throughout this paper refers to a simulated altitude in a low pressure chamber.

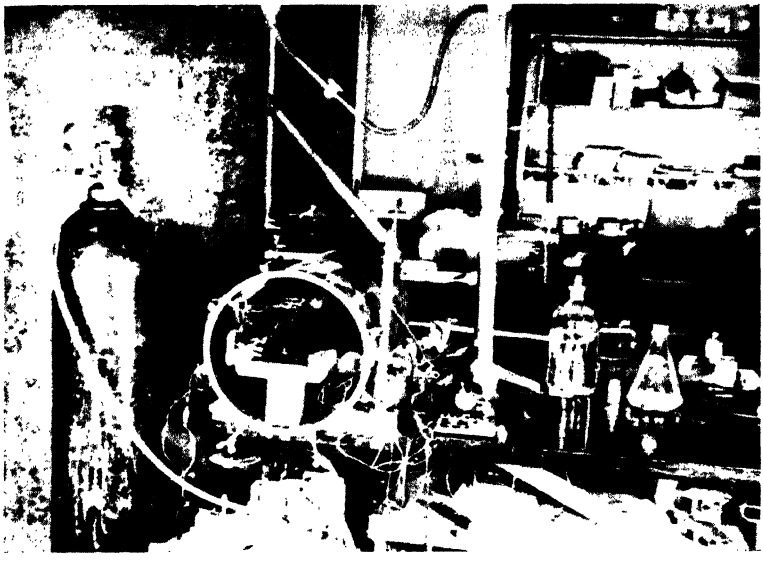
when the blood is drawn at an altitude of 45,000 feet or during a prolonged exposure to an air pressure of 100 lb./in.^{2}, bubbles have not appeared at the vapor pressure except in a few instances where contamination was suspected. These experiments indicate that all the formed elements of the blood (red and white corpuscles, platelets, fat globules or blood dust) play no part in bubble formation, that air masses do not normally pass from alveoli to lung capillaries (although they may when the alveolar air pressure is raised above that in the capillaries) and that movement of the blood with turbulence and vortex formation around the valves of the heart does not normally start bubble formation. They lead to the conclusion that bubbles must arise from gas nuclei sticking to or formed on or within the endothelial linings of the vascular system or extravascular spaces and only when they have enlarged to the point of instability do they pass into the blood stream.

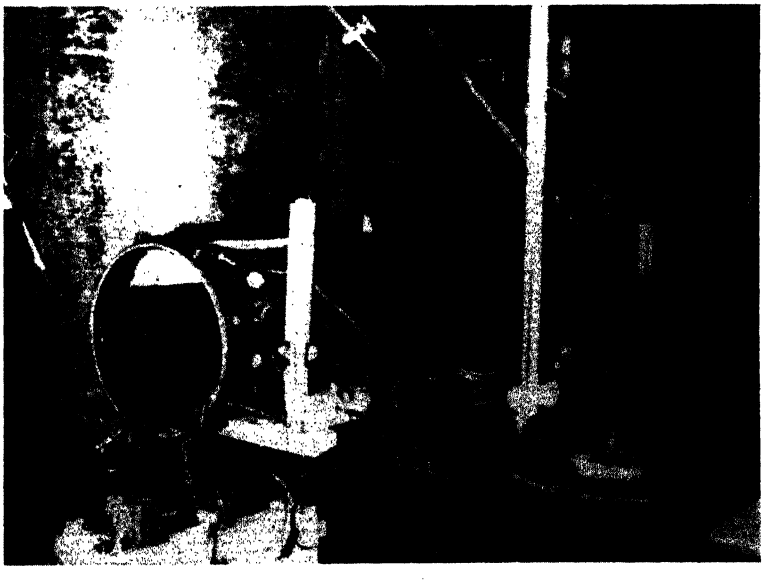
A few attempts were made to sample venous blood from the postcava below the renal vein, but the low blood pressure and the tendency for this vessel to pass into spasm has introduced difficulties. These can be overcome but the postcaval wall is so thin that bubbles within can be readily seen with the naked eye. The postcava, in fact, is a natural pompholygometer for testing blood from the hind quarters of the body and has been used in our standard experimental procedure in studying bubble formation.

ANIMAL TECHNIQUE

The cat is anesthetized with nembutal and the abdomen opened with little loss of blood. The viscera are pushed aside and covered with moist cotton to prevent evaporation, thereby exposing a considerable length of the postcava together with its tributary branches. When the cat is placed in a horizontal position in the

FIG. 6. Above—View of high altitude chamber with cat in position, reservoir vacuum tank for rapid removal of air from altitude chamber, oxygen cylinder, mercury altitude gauge and accessory apparatus. Below—Near view of same, showing mercury blood pressure manometer on outside of altitude chamber. Equalization of pressure is effected by connecting open end with interior of chamber.





altitude chamber and well illuminated, observation can be readily made through the glass observation ports, and bubbles of 0.2 mm. diameter can be seen within the veins. Only when bubbles are very large can they be detected in arteries.

The altitude chamber, shown in Figure 6, is an old autoclave, connected by a good-sized pipe and valve to a large reservoir

FIG. 7. Various devices for manipulation in tank. Upper left—artery clamp closed by electromagnet. Lower left—artery clamp opened by electromagnet. Upper right—mouse trap spinal cord cutter. Lower right —motor and eccentric for passive movement of leg.

chamber which can be evacuated with an air pump. The capacity is such that by opening the valve quickly or slowly the small chamber reaches the simulated altitude at any desired rate from explosive decompression to our usual average "rate of climb" of 5,000 feet per second.

The chamber is too small to admit a man and the altitudes are often so high that no man could exist for any length of time.

Therefore, various devices have been developed for physiological manipulation from without the chamber. Some of these devices are shown in Figure 7. Blood pressure can be determined with a carotid cannula inside and a mercury manometer outside the chamber, provided care is taken to remove gas nuclei from connecting tubes. The open end of the manometer is reconnected to the altitude chamber for equalization of pressure as shown in the lower portion of Figure 6. Electromagnetic clamps for blood vessels or tracheae have been devised that will either open or close on making a contact, as well as an automatic syringe for injecting solutions. Finally, an ingenious "mouse trap sectioner" for cutting the spinal cord with the cat in the chamber is activated by electromagnetic release of the spring of the trap to which a wire is attached that cuts through the cord.

Pure oxygen is administered by a glass tube fitting loosely over a tracheal cannula. The altitude has been mostly 45,000 feet (110 mm. Hg), selected as a convenient high level at which cats would live for at least an hour with oxygen breathing despite considerable anoxemia. Under these conditions the rate and extent of bubble growth would be as great as possible.

RESTING ANIMALS AT ALTITUDE

If a series of resting nembutal anesthetized cats are prepared in this manner and observed for at least an hour at 45,000 feet, bubbles rarely appear in the postcava. Even at 50,000 feet where a percentage of the animals die, bubbles are not usually found during a 60-minute observation or during the life of the cat. Since dissolved gas in the animal is constantly and rapidly lost at this altitude by the efficient breathing and circulatory mechanisms, aided by further compensatory changes resulting from anoxemia, experiments were undertaken to retain the dissolved gas, expecting that bubbles might then appear. Both arteries (aorta and collateral arteries) and veins (postcava) were tied, or the leg was tourniquetted or the trachea clamped (when the heart continues to beat for 6–8 minutes), but even under these more favorable conditions, bubbles failed to appear.

ANIMALS STIMULATED AT ALTITUDE

However, if the hind legs of the cat were stimulated vigorously, bubbles from limb veins could be observed moving up the postcava, sometimes within 5 seconds of the beginning of stimulation. By 16 minutes the great majority of the animals had formed bubbles although there was variation in time of appearance from cat to cat and an occasional animal was resistant to bubble formation and remained free of them despite this procedure. If only one leg was stimulated, the bubbles formed in that leg and appeared in veins draining that leg only. This important discovery of the effect of muscle contraction on appearance of bubbles was first made by Whitaker, Blinks and collaborators,[8] using bullfrogs, and has its practical application in the well recognized fact that straining movements and exercise among aviators is the most important predisposing factor for the development of bends and chokes.

Another point of special interest is the variability in time of bubble formation in cats, which has its counterpart also in the varying resistance and susceptibility to decompression sickness observed in men. Part of this variability may be due to force and extent of muscle contraction on stimulation, part to differences in the circulation and part to the fat content of the animals, for it is well recognized that fat men are particularly susceptible to bends after diving and Boycott and Damant (8) found a correlation between fat content and bends in goats after an exposure to compressed air. Since nitrogen is about five times more soluble in fat than in water, a large volume of gas is available for growth of bubbles in fatty regions.

To test the effect of fat content and bubble formation at altitude, the fat of the subcutaneous region below the diaphragm (including the inguinal fat pads and deposits below the skin of the legs as well as the popliteal space) was removed from more than 400 cats, weighed and then expressed as a percentage of the body weight. When time of bubble appearance was plotted vs. fat content, no correlation could be detected, a result we be-

[8] Private communication.

lieve to be connected with the rather rapid appearance of bubbles after the muscles are stimulated. Only when the bubble formation is relatively slow, as on decompression from critical excess air pressures, have we observed a correlation between time of bubble production and fat. It is under these conditions that the slow seeping of additional nitrogen from fat deposits can make itself felt. Fat does not appear to be a primary cause of variability.

If stimulation of the hind legs is made at 35,000 feet (180 mm. Hg) bubbles also appear but they are smaller and often hard to see. At lower altitudes we may expect to find minute bubbles of microscopic dimensions too small to do any harm.

ANIMALS STIMULATED BEFORE ASCENT

Evidently stimulation of the legs at altitude increases the ΔP above that due to the altitude itself, so that bubbles appear in consequence. Such an increase in ΔP must enlarge gas nuclei even at ground level, and it is conceivable that the enlarged nuclei might persist for a sufficient time to give rise to bubbles if the animals were subsequently exposed to high altitude. We have a comparable experiment in a glass tube filled with water and free of any gas nuclei that can grow to visible bubble size at 45,000 feet (110 mm. Hg). If the tube is hit a few blows at ground level, no bubbles may be visible but when immediately evacuated to 110 mm. Hg. bubbles appear. If there is a delay before evacuation the gas nuclei disappear and bubbles will not form.

The analogous experiment succeeds in the animal. When the hind legs of cats were stimulated once a second for twenty seconds and the animals immediately taken to 45,000 feet, bubbles appeared in 7 out of 10 cats. However, if there was a wait of ten minutes before ascent to 45,000 feet, in only 2 of 10 cats did bubbles appear. During this wait the enlarged gas nuclei in the animals had returned to their original condition. It is therefore apparent that vigorous muscle contraction and straining movements should not be carried out just before ascent, because, due

to the excess ΔP, gas nuclei will be enlarged and may form bubbles at the altitude.

On the other hand, muscular exercise must have a beneficial effect in preventing bubble formation by virtue of the marked vasodilation and opening of closed capillaries in muscle tissue as well as the increased circulation and hyperventilation necessary rapidly to remove carbon dioxide from the blood. During exercise exactly those physiological mechanisms, naturally designed to remove a gas (carbon dioxide), are brought into play and they must be just as efficient in removing a gas such as nitrogen which is necessary for permanent bubble formation. The relative influence of these two opposing factors—the enlargement of gas nuclei by muscle contraction, and the increased gas elimination due to similar activity—will determine how much and how strenuous the exercise can be.

Actually, experience has shown that in man pre-exercise at ground level before ascent, even when combined with oxygen breathing, is not as beneficial as might be expected and little increase of protection is afforded over inhalation of pure oxygen at rest unless the exercise ceases some time before ascent. Moreover, the hyperventilation and additional oxygen required to make up the oxygen debt imposes an excessive strain on the present oxygen mask and breathing equipment. In cats prestimulation with continued stimulation during ascent and at altitude does so increase the circulation and accelerate removal of nitrogen that visible bubbles are rarely observed in their blood.

In decompression after deep diving or other work under pressure, exercise has been advocated as a ready and efficient means of accelerating the removal of nitrogen. There are in fact reasons why this increase in ΔP from exercise is much less effective at ground level than at high altitude, reasons bound up with the mechanism of bubble formation during muscle contraction.

MECHANISM OF BUBBLE FORMATION AFTER MUSCLE CONTRACTION

It is obvious that an increase in ΔP, which we have defined as the gas tension, t, minus the hydrostatic pressure, P, may re-

sult either from an increased gas production or a decreased hydrostatic pressure, due to stretching of the liquid, i.e., to mechanical tension. Both factors are involved in muscle activity, the excess carbon dioxide and the mechanical tension of contraction. One of these factors, the decreased *P*, becomes particularly important at altitude because the animal is already near the vapor pressure of water (47 mm. Hg) and the mechanical tension of muscle contraction may expand a gas nucleus to a large vapor cavity such as is shown in Figure 4. Dissolved gases would diffuse into this cavity at a rate depending on their relative concentrations and diffusion coefficients and the surface area of the cavity. The larger the vapor cavity, and the longer it persists before collapse, the larger will be the resultant bubbles. In previously air pressure treated cats at one atmosphere, with the same mechanical tension developed by muscle contraction, the decreased pressure may not reach the vapor pressure or may be only slightly below the vapor pressure so that a vapor cavity would be small and its persistence short. In this case the gas diffusing into the cavity would be far less; bubbles would be small and take more time to reach a visible size. In either pressure exposed animals or those at altitude, the high concentration of newly formed carbon dioxide from muscle contraction must greatly accelerate the early growth of bubbles.

Our conception of the mechanism of bubble formation during muscle contraction emphasizes both carbon dioxide concentration and mechanical tension, but, if the above reasoning is correct, the facts point to mechanical tension as the more important of the two factors. Additional evidence in favor of mechanical tension comes from two types of experiment. In one, the legs of cats have been stretched without active muscle contraction and hence without carbon dioxide production, and in a second, lactic acid was injected so as to increase carbon dioxide without the mechanical tension. In a third type of experiment the muscles of the legs have been crushed and injured. The crushing must itself exert a mechanical tension on muscle fibers as well as mechanically stimulate them locally, with carbon dioxide produc-

tion and acid of injury. Although its bearing on the theory of bubble formation during muscle contraction is not so clear cut, it has turned out to be a most effective method of inducing bubble formation. These three types of experiment may now be considered.

CRUSHED AND INJURED TISSUES

The first experiments on injured tissues were carried out by striking the thigh muscles of the anesthetized cat with a rubber hammer so as not to puncture the skin but to bruise and break small vessels, causing extravasation of blood in muscle tissue. The animals were then immediately taken to 45,000 feet altitude and in 9 out of 10 cats bubbles appeared in the postcava.

The blows might have resulted in bubble formation by setting up a series of pressure pulses, as in the glass tube experiments previously described (p. 50). Therefore a crushing device was constructed for slowly squeezing the thigh muscles and actually tearing them, but without perforation of the skin, which might allow air to enter from outside. Again, when cats so treated were taken to 45,000 feet, either immediately or after a wait of 10 minutes at ground level, the bubble formation was profuse. Bubbles could also be found locally in the injured region. These experiments indicate that bubbles might readily form and pass into the circulation of injured men at a high altitude.

The crushing procedure is such that very strong stretching forces are exerted on the muscle fibers, sufficient in some cases to actually break them transversely. This mechanical pull must play an important part in the bubble production, for it can be shown that injury to tissue containing no striated muscle is ineffective in forming bubbles, even when the volume of tissue injured is greater than in the muscle injury experiments. Thus, stripping the intestines, squeezing the kidney to a pulp within its capsule, crushing the testes or bruising large areas of the skin have all failed to result in bubble formation when the cats were observed at 45,000 feet. Acid of injury and carbon dioxide must be formed in these tissues also (although possibly not in such high concentration as in muscle) so that the mechanical tension

developed in crushing the muscle tissue would appear to be the important factor in formation of bubbles during muscle injury.

PASSIVE MOVEMENT AND STRETCHING

Passive movement of a leg can be attained by attaching the foot to an eccentric run by a motor, so that the leg is rapidly moved back and forth. Movement of this sort at an altitude of 45,000 feet does not give rise to bubbles. However, if a wire is attached to the ankle of a resting anesthetized cat, securely tied to its operating board, and the leg vigorously stretched at 45,000 feet, bubbles will appear, provided the blood supply to the leg is cut off by clamping arteries and veins. Without the vessel clamping, removal of gases by blood flow is sufficiently rapid to prevent bubble formation. Since the clamping of arteries and veins does not give rise to bubble formation without stretching, it appears probable that the mechanical tension is the cause of the bubbles.

LACTIC ACID INJECTION

Any local increase in lactic acid should liberate carbon dioxide from blood and imitate the excess carbon dioxide produced on muscle contraction. Therefore gas nucleus-free lactic acid was injected into the aorta, usually under conditions (clamping of proper vessels) that prevented its rapid removal by the circulation but insured its distribution to the hind quarters of the cat. These experiments have been carried out, both on cats previously exposed to air pressures of a critical value and duration (3.5 atmospheres for 2 hr.) such that bubbles ordinarily appear only a considerable time after decompression, and also in cats at an altitude of 45,000 feet.

In the cats exposed to compressed air collateral vessels were not clamped. The injection was made immediately after decompression and no bubbles were observed. In the "altitude" cats, injection was made just before a rapid ascent to 45,000 feet. In these animals bubbles appeared in 9 out of 21 animals whereas in a control series injected with gas nucleus-free saline solution only 1 out of 10 formed bubbles. The difference is not signifi-

cant when analyzed by the method of Chi square, but there is perhaps a trend, a tendency for lactic acid to favor bubbles, but not nearly as significant as the effect of muscle contraction itself. These experiments, combined with the leg stretching experiments, indicate that mechanical tension must be the predominant factor in the formation of bubbles.

COMPOSITION OF BUBBLES IN BLOOD

Analyses of gas bubbles in blood by the ordinary methods indicate a very high percentage of nitrogen. They have been considered nitrogen bubbles for all practical purposes. This is what might be expected from the tensions of gases at equilibrium in blood. However, when gas moves into a bubble rapidly, particularly into a rapidly forming vapor cavity, the composition of the gas left after collapse will depend mostly on the concentration of gas dissolved in the surrounding liquid, i.e., its solubility at a given tension. Highly soluble carbon dioxide must be present in excess. If the bubbles could be obtained and analyzed while very small the carbon dioxide content would undoubtedly be high. This excess carbon dioxide must pass out of the bubble again and the final composition depend on gas tension rather than concentration. Therefore, in the early growth of a gas nucleus carbon dioxide may be very important, but for bubble persistence the nitrogen tension, represented by a partial gas pressure, is the determining factor.

When an animal breathes pure oxygen the nitrogen is flushed out of the body, but the carbon dioxide, which is a body product, remains at practically its original level. Carbon dioxide is also constant in an animal exposed to high air pressure, which dissolves an excess of nitrogen but the increased carbon dioxide pressure in the compressed air is small compared to the carbon dioxide produced by the body. Oxygen is always so rapidly used by the body that its tension is kept relatively low. Nitrogen becomes the important gas for permanent bubble formation and its removal essential.

THE EFFECT OF OXYGEN BREATHING

It is therefore not surprising to find that oxygen breathing for a sufficient time before ascent will completely prevent the formation of visible bubbles in the postcava of cats after vigorous stimulation of the hind legs. The actual time of oxygen breathing depends on the strength of contraction. With our standard 17-volt, 60-cycle, A.C. stimulus, one hour is sufficient to protect half the cats but one-half hour is not nearly enough. If the stimulus is reduced to 6 volts, resulting in a less vigorous muscle contraction, the cats are fully protected after one-half hour of oxygen breathing.

A small stimulus will result in the development of a lower tension, both locally, due to fewer fibers pulling together at one spot, and generally, due to fewer muscles coming into play. The end result is reduced hydrostatic pressure, which does not give a sufficient ΔP for bubble formation at the low nitrogen tension existing after one-half hour of breathing oxygen. The greater number of muscle fibers contracting after strong stimulation will produce more carbon dioxide but because of the circulatory adjustments bound up with excess carbon dioxide production, the concentration of this gas will not rise proportionally. The decreased hydrostatic pressure due to mechanical tension again appears to be more important than the carbon dioxide. After one-half to one hour of oxygen breathing the nitrogen tension in the postcaval blood of cats is very low so that only gas in the most inaccessible and poorly vascularized places can be involved in bubble production.

In man also, oxygen breathing before ascent will completely prevent the appearance of all symptoms of decompression sickness, and offers the most effective prophylactic treatment. In man, the amount of oxygen breathing necessary has also been shown to depend on the extent of exercise at altitude. The actual time of oxygen breathing to prevent bends in man and to prevent bubble formation in the blood of cats is about the same.

Moreover, a comparison of nitrogen elimination curves from

analysis of nitrogen tension in veins and arteries of man and cat show a surprising similarity in the actual time relations, despite the huge difference in volume of man and cat (30:1). In both animals also, the rate of nitrogen elimination during exercise is greatly increased. The circulatory systems of man and cat appear to be so adapted that in man oxygen can be supplied and waste removed in approximately the same time as in the cat, despite the difference in volume of tissue to be serviced. This adaptive relation is probably widespread among mammals of different mass and is no doubt connected with some basic property of cells, perhaps the time that certain cells can withstand lack of oxygen or the accumulation of injurious metabolites.

THE EFFECT OF CIRCULATORY AND RESPIRATORY CHANGES

We have already seen that the circulatory and respiratory changes of exercise are perfectly adapted for rapid removal of gases but at the same time the muscle contraction of exercise, even at ground level, is an effective producer of bubbles. Some other method of increasing the circulation would be desirable to reduce the incidence of bends.

One, the use of drugs has not been thoroughly tested in the cat. Another, lack of oxygen, cannot be successfully applied to man but experimentally, in the cat, has turned out to be a fairly efficient method of reducing the bubble formation. When oxygen is withheld from the animals at 45,000 feet for some minutes before stimulation is begun, there is a significant difference in the number of cats in which bubbles appear, as compared with a control series receiving oxygen continuously. The hyperventilation of these anoxemic animals is observed to be marked and we may presume that accompanying the hyperventilation there is the usual reflex vasodilation designed to supply muscle tissue with all the oxygen available. At the same time nitrogen is effectively removed.

THE SITE OF BUBBLE FORMATION

The wide variety of decompression sickness symptoms has led to the view that bubbles may form in almost any part of the

body, and indeed, after an extreme compressed air treatment, bubbles are found in all regions of a cat, including arteries and veins, lymph vessels, eye humors and amniotic fluid, although not in the bladder urine. They are very abundant in fatty tissue and can be seen in veins draining fat deposits.

We have not determined whether these bubbles, which are abundant in the viscera, ever occur *within*[9] the cells of the body but this is very unlikely. All attempts to detect bubble formation within single living uninjured cells (such as Paramecia, Amoeba, Nitella, sea urchin or starfish eggs) have failed. In some experiments the cells were saturated at a nitrogen pressure as high as 2,300 lb./in.^{2} and then decompressed. The outer surface of the cells was observed to act as a source of bubbles, but they never formed within, unless the cell was injured or dead. It is therefore doubtful that bubbles form within mammalian cells but they probably appear in intercellular spaces and would be most likely to form where one surface can be pulled away from another.

In cats at an altitude of 45,000 feet, bubbles, after stimulation, are confined to the blood vessels and occasionally appear in lymph vessels. They are mostly found in veins, probably due to the lower blood pressure and greater gas tension and hence lower ΔP, as well as direction of flow. Bubbles have never been observed in eye humors, urine or amniotic fluid, nor have they been observed around joints, although examination could not be made at altitude. At ground level this gas may have contracted to so small a volume as to escape detection.

In man numerous X-ray studies made in altitude chambers have disclosed the presence of air masses in joint cavities, in popliteal fat and in the fascia between muscles. All observers agree that air may be present in these regions without the pain of bends. It has also been observed that fluid can be injected into the bursa without giving rise to discomfort and gas in the bursa itself is not correlated with pain. Statistically, gas in

[9] Gersh (20) has described bubbles within fat cells from guinea pigs (previously exposed to air-pressure treatment), examined in sections prepared by the rapid-freezing technique.

popliteal fat and muscle fascia appears most likely to cause pain and we are led to the view that the undue expansion of gas in critical sensitive regions of connective tissue[10] stimulates sensory endings of pain nerves. Since localization of pain is not usually precise, it is frequently referred to the joint.

Another theory of bends pain refers it to the pain of muscle contraction in absence of oxygen, described by Sir Thomas Lewis (21). Bubbles blocking the blood flow through muscle (true aeroembolism) would prevent access of oxygen and initiate conditions for the pain of anoxic contraction. However, the above mentioned X-ray photographs do not with certainty reveal bubbles caught in blood vessels. This fact, together with the observation that local pressure application or even a blocking of the blood flow by tourniquet above the painful region (which may also increase pressure by blocking venous return) will relieve bends, are both contrary to the intravascular embolism theory. They favor extravascular gas as the cause of bends pain.

Tension must be involved in gas formation in joints. Even at ground level some persons can develop gas in the shoulder joint by a proper rotary motion of the arm that puts strain on the shoulder. By a sudden pull on the fingers also, gas will appear in the finger joint as demonstrated by Nordheim (22). At high altitude the formation of gas under such conditions will be greatly facilitated.

It is impossible, from examination of the chest by roentgenogram, to distinguish excess gas in blood vessels from air in lung alveoli but several cases of chokes have shown an enlargement of the right heart which could be due to gas blocking the normal blood flow.[11] As shown by Van Allen, Hrdina and Clark (23) and confirmed by our work on the cat, bubbles do not ordinarily pass the lung capillaries, due to the low pulmonary blood pressure, although they are able to pass systemic capillaries. We have frequently observed the heart of a cat to be distended with air from a rather copious bubble formation in veins when no

[10] Dr. A. C. Ivy informs me that he has a number of cases of bends where no gas could be detected in the roentgenograms.

[11] Private communication from Dr. Ivy.

bubbles could be found in arteries. It seems highly probable that air collecting in pulmonary capillaries could give rise to those sensations characteristic of the chokes.

Nervous symptoms are more difficult to analyze. They could be due to gas blocking capillaries of the central nervous system or to gas in nerve sheaths exerting pressure on nerve trunks. We do not know. Skin effects are also puzzling. Local bubbles in the skin have not been demonstrated. Frequently the skin rash will be distributed in a cutaneous area supplied by one sensory trunk, like the dermatome areas of herpes zoster. Possibly skin rashes, as well as the local anesthesias and hyperesthesias, are the result of nerve blockage, either central or peripheral. Some details remain to be filled in but on the whole the etiology of decompression sickness can best be expressed by one word—bubbles.

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PRODUCTION AND NATURE OF ANTIBIOTIC SUBSTANCES[1,2]

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"Entrainé, enchainé devrais je dire, par une logique presque inflexible de mes études j'ai passé des recherches de cristallographie et de chimie moléculaire a l'étude des ferments."—L. Pasteur.

INTRODUCTORY

WHEN Pasteur first turned from his purely chemical studies on crystals to the field of microbiology, he little visualized the manifold developments of the new subject that were to result: microorganisms as causative agents of disease, their rôle in fermentations, in the preparation and in spoilage of beverages and foodstuffs and in agriculture, and finally as agents for combating disease. This newest branch of microbiology, the study of the production and nature of antibiotic substances and their utilization as chemotherapeutic agents is not of recent origin, but it is only comparatively recently that it has aroused universal attention.

Bouchard in 1889 and Emmerich and Low in 1899 (1) demonstrated that certain bacteria belonging to the *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*) group have the capacity of inhibiting the growth of other bacteria and of actually killing these organisms. This was found to be due to the production of a substance designated as pyocyanase, the first antibiotic substance ever described. However, this phenomenon was looked upon rather as a freak, the substance being considered as an enzyme that had the capacity of bringing about the lysis of certain bacterial cells. The plant pathologist has been constantly led to observe, in his cultures of fungi and bacteria, the detrimental

[1] Lecture delivered November 16, 1944.

[2] Journal Series paper of the New Jersey Agricultural Experiment Station, Rutgers University, department of Microbiology.

action of one organism upon another, but he was satisfied to designate this phenomenon as one of "staling," without attempting to uncover the manifold reactions involved. The soil microbiologist, in his studies of mixed populations of microorganisms, long recognized the fact that one organism may affect to a marked degree the activities of another, but he dealt with a highly heterogeneous medium, the soil, and his problem appeared hopelessly complex. By designating as "soil toxins" the injurious substances thus produced he failed to succeed in unravelling further the complicated reactions involved. The chemist isolated and even crystallized certain antimicrobial substances produced by bacteria, such as pyocyanin, and by fungi, such as penicillic acid and gliotoxin, but the practical utilization of these substances was hardly visualized. Only isolated investigators recognized the great possibilities involved in the antagonistic interrelations among microorganisms and drew attention to their possible application. This was first done by Pasteur and Joubert in 1877, followed by Cantani in 1885, by Emmerich and Low in 1899, by Vaudremer in 1913, by Much in 1924, by Fleming in 1929, and finally by Dubos in 1939.

It remained for the last half decade to uncover the great potentialities of this highly important field of microbiology, with its many phases touching upon chemistry, physiology and chemotherapy.

NATURE OF ANTIBIOTIC SUBSTANCES

Antibiotic substances possess certain chemical and biological properties which distinguish them from the common antiseptics and disinfectants. These can be summarized as follows:

1. They are produced by living organisms, often designated as antagonists because of their ability to inhibit the growth of or to destroy bacteria and other microorganisms. Certain purely synthetic compounds have antibacterial and antifungal properties similar to those of typical antibiotic agents.

2. Their action is primarily bacteriostatic in nature, bringing about inhibition of growth; their bactericidal properties may also, however, be very pronounced.

TABLE I

Bacteriostatic Action of Several Antibiotic Substances upon Different Bacteria (14)

Activity in dilution units per gram of material

Substance	Test organism					
	S. aureus	*M. phlei*	*B. subtilis*	*E. coli*	*Ps. aeruginosa* I	*Ps. aeruginosa* II
Penicillin	5,000,000	7,500	19,000,000	< 5,000	0	0
Chaetomin	400,000	800,000	400,000	0	0	0
Clavacin	50,300	67,000	65,000	100,000	5,000	7,500
Fumigacin	303,000	91,000	200,000	0	0	0
Actinomycin	6,000,000	4,000,000	60,000,000	15,000	0	0
Streptothricin	500,000	300,000	1,000,000	100,000	1,000	5,000
Streptomycin	330,000	2,000,000	330,000	100,000	10,000	20,000

3. They are selective in their action upon bacteria, some affecting largely Gram-positive and only to a limited extent Gram-negative forms, whereas others act upon certain specific bacteria within each of these groups, and only to a limited extent or not at all against other bacteria. These differences are qualitative as well as quantitative; hence one may speak of a "bacteriostatic" or "antibiotic spectrum," namely the selective antibacterial or antimicrobial action of an antibiotic substance (Table I).

4. The effect of the substrate upon the antibacterial activities of different antibiotic substances varies greatly. Some substrates have no effect at all upon a specific substance; others reduce the activity of the substance, due to the neutralizing effect of some constituent, such as peptone, glucose or serum; some, like blood, may adsorb the substance, thus completely inactivating it; some substances require for their activity the presence in the medium of specific constitutents, such as glucose, in order to become effective.

5. Antibiotic substances vary greatly in their chemical nature. A large number of compounds have already been isolated, although only comparatively few have so far been crystallized (Table II).

6. Antibiotic substances vary greatly in their toxicity to animals, some being highly toxic, others having virtually no toxicity at all, and most others falling between these two extremes. They also vary in their *in vivo* activity.

7. The mechanism of antimicrobial action of various antibiotic substances is different. Some interfere with the growth of bacteria and their cell division, others with some phase of bacterial respiration and still others with the utilization of essential metabolites by the bacteria.

8. Many antagonistic organisms produce more than one antibiotic substance. *Pseudomonas aeruginosa* has long ago been known to produce two agents, pyocyanase and pyocyanin, distinct from one another both chemically and in their antibacterial action. *Bacillus brevis* produces gramicidin and tyrocidine;

Penicillium notatum forms penicillin and notatin; *Aspergillus flavus* produces aspergillic acid and flavicin; *A. fumigatus* is capable of forming four substances, namely spinulosin, fumigatin, fumigacin and gliotoxin. The culture filtrate of an organism producing an antibiotic substance often differs, therefore, in its antimicrobial activities from that of the isolated substance.

9. Some substances are produced by different types of organisms. Penicillin and penicillin-like compounds are formed by many different strains of *P. notatum, P. chrysogenum, A. flavus, A. giganteus, A. parasiticus,* and other fungi. Gliotoxin is produced by species of *Trichoderma, Gliocladium,* and *A. fumigatus.* Clavacin (claviformin, patulin, and clavatin) is formed by a large number of fungi, including *A. clavatus, P. claviforme, P. patulum* and *Gymnoascus.* Considerable confusion may thus result from the duplication of names for the same substance.

TABLE II

Chemical Formulae of Antibiotic Substances That have been Crystallized or Highly Purified

Bacteria and actinomycetes	Fungi or molds
Gramicidin—polypeptide	Penicillin—? ? ? ? ?
Tyrocidine—polypeptide	Gliotoxin—$C_{12}H_{14}N_2O_4S_2$
Pyocyanase—lipoid	Chaetomin—$C_{12}H_{16}N_2O_6S$*
Pyocyanin—$C_{13}H_{10}N_2O$	Aspergillic acid—$C_{12}H_{20}N_2O_2$
Iodinin—$C_{12}H_8N_2O_3$	Fumigacin—$C_{20}H_{38}O_7$
Actinomycin—$C_{41}H_{56}N_8O_{11}$*	Citrinin—$C_{13}H_{14}O_5$
Proactinomycin—basic compound	Clavacin—$C_7H_6O_4$
Streptothricin—basic compound	Penicillic acid—$C_8H_{10}O_4$
Streptomycin—basic compound	Kojic acid—$C_6H_6O_4$
Actinomycetin—protein	Fumigatin—$C_8H_8O_4$
	Puberulic acid—$C_6H_6O_6$
	Puberulonic acid—$C_5H_4O_6$

* Approximate.

10. The same type of substance, when produced by different organisms or even by the same organism grown on different

media or under different conditions, may show variation, both in its chemical nature and in its antibiotic spectrum, from those of the established type.

ANTAGONISTIC PROPERTIES OF MICROORGANISMS

The production of antibiotic substances is a phenomenon widely distributed among living systems, and is not limited to microorganisms. However, the latter, comprising certain fungi, bacteria and actinomycetes, are now recognized to be the all-important sources for the production of such substances. These microorganisms are often spoken of as antagonistic or as possessing antagonistic properties.

The quantitative formation of an antibiotic substance, and often its very nature, are controlled by the nutrition and conditions of growth of the antagonistic organism. One type of substance may be produced by the same organism when grown in surface culture and another under submerged conditions: *A. flavus,* for example, produces largely aspergillic acid when grown in stationary culture, and flavicin, a penicillin-like substance, when grown in submerged culture. The nutrition of *B. brevis* and the production of tyrothricin by this organism are quite different when it is grown under submerged as compared with stationary conditions. Certain organisms require growth-promoting agents for the formation of a specific substance, such as penicillin and streptomycin, whereas other organisms do not.

Attempts have been made to explain the capacity of antagonistic microorganisms to produce antibiotic substances on the basis of their struggle for existence in nature. The existing evidence does not fully justify this assumption. Although the presence in the soil of certain toxic compounds, apparently of the antibiotic type, has been demonstrated (6, 16), no evidence has as yet been submitted to prove that the accumulation or even the formation by microorganisms of typical antibiotic substances under soil conditions is based upon competition for either nutrients or space, or both.

The production of penicillin by *P. notatum,* a common soil fungus can serve as a typical example. This compound is

highly labile and is subject to destruction by various bacteria inhabiting the soil in great abundance. Penicillin is formed by the fungus in purely artificial media and under special conditions of culture. One could hardly visualize that a substance, so readily destroyed by a great variety of bacteria as penicillin is, could be produced in the soil in sufficient amounts to influence the growth of bacteria and especially of fungi, with which *P. notatum* has to compete for food and for space. How could the ability of this organism to produce penicillin in artificial media affect its survival in as complex an environment as that of a natural soil? Various fungi, such as species of *Mucor, Rhizopus* and other Mucorales, that do not form either penicillin or any other antibiotic substance, so far as is known at present, may be found in the soil much more abundantly than *P. notatum.* From the point of view of nutrition of fungi, penicillin appears to be of little importance to *P. notatum.*

Trichoderma, another common soil fungus, has the capacity of producing, under certain conditions of culture, the antibiotic substance gliotoxin. This organism is capable of attacking and destroying in the soil the important plant pathogen *Rhizoctonia* (17); however, one can hardly be justified in suggesting that *Trichoderma* depends upon *Rhizoctonia* as the sole nutrient for its existence in the soil or even that it competes with it for any particular place or nutrient in the soil. Of these two organisms, *Trichoderma* is much more omnivorous and can derive its nutrients from a great variety of chemically pure substrates, namely, cellulose, hemicelluloses, and proteins, whereas *Rhizoctonia* depends to a considerable extent for its food upon the plant that it attacks, and is apparently much more selective in its mode of nutrition. In order for *Trichoderma* to produce sufficient amounts of gliotoxin in artificial culture, special conditions of growth and nutrition are required. These conditions tend to favor in the organism certain physiological mechanisms, that are markedly different from those manifested in a natural environment.

Even those antibiotic substances that are more stable in nature than either penicillin or gliotoxin, and that may, therefore,

be formed and even accumulate in the soil, will often be neutralized in their antibacterial action in that environment. This is true of actinomycin, an antibiotic substance which is quite inactive in a reduced state, which is likely to become reduced in the soil, and which is also inhibited in its antibiotic action by the humus constituents of soil. It is true of gramicidin, a substance inhibited by phospholipins, compounds found abundantly in the soil organic matter.

If one were to try to find justification for every biochemical capacity of an organism, what possible reason could there be for the ability of *Clostridium botulinus, Cl. welchii* and *Cl. tetani,* three soil-inhabiting bacteria, to produce powerful animal toxins that can have no possible effect on other bacteria or fungi living in close proximity with them?

The teleological concept comprising the argument as to the "purposefulness" vs. "purposelessness" of certain biological reactions appears to verge on some of the vitalistic ideologies. It is easy to understand that an organism living in a certain medium has a given biochemical property, as the ability to decompose cellulose, when the medium happens to be a compost rich in cellulose. However, it is not at all easy to appreciate the apparently normal life of the same or similar organisms in another natural substrate, such as the sea, where comparatively little true cellulose is ever found. The ability of some bacteria or fungi to produce specific enzymes in response to certain constituents in the medium may appear as purposeful, but what purpose could there be for an organism to possess mechanisms for which it apparently has no use whatsoever in a given environment?

Further, there is a marked variation in the presence and relative abundance, in soils or in water basins, of microorganisms that are capable of producing specific antibiotic substances. Bacteria producing tyrothricin, as well as fungi capable of forming penicillin or clavacin are very abundant in the soil. Other organisms producing antibiotic substances can be isolated from the soil, however, only after very diligent search: this is true of *Actinomyces antibioticus* and *A. lavendulae,* pro-

ducers of actinomycin and streptothricin, respectively. In spite of the fact that 20–40 per cent of all soil actinomycetes possess the capability of inhibiting the growth of various bacteria, strains of the two actinomyces organisms were isolated only twice in our laboratory between 1940 and 1944. This rare occurrence of these two particular organisms is especially interesting, since during this period nearly 1,000 cultures of actinomycetes were tested for their production of antibacterial agents. It may even be of greater interest that the second isolates of both strains showed certain marked differences from the corresponding original strains; these differences were either quantitative, as in the case of actinomycin, or qualitative, as in the case of streptothricin, the new strain yielding a substance that had a somewhat different antibacterial spectrum from that of the old one.

These and other observations tend to emphasize the fact that although microorganisms capable of producing antibiotic substances are widely distributed in nature, only a few groups have the inherent capacity to form these substances in significant amounts under special conditions of culture. Some strains produce a given substance under one set of conditions, whereas other strains produce the same substance or a closely related compound under other conditions, as in stationary vs. submerged and agitated cultures. This speaks further for an inherent property of certain organisms to produce substances that have the capacity to inhibit the growth of specific bacteria, a property that has nothing whatsoever to do with the survival of such organisms in nature.

One need not deny the fact that certain microorganisms are capable of exerting antagonistic effects in the soil itself. It remains to be determined, however, whether this is due to the production by these organisms of antibiotic substances or to totally different mechanisms, as competition for food or the production by one organism of an environment unfavorable to the other. Nakhimovskaia (9) demonstrated that the nature of the soil and its treatment are of considerable importance in this connection;

antibacterial action is more intense in light than in heavy soils. The addition of organic matter either depresses these effects in the soil, as in the case of actinomycetes, or it favors the development of antagonistic fungi, which bring about the control of certain plant pathogens.

The isolation and cultivation of antagonistic pathogens for the purpose of producing antibiotic substance thus represent fundamental problems in physiology and biochemistry, especially the response of these organisms to nutrition and environment. Further information concerning the ecology of these organisms may be gained from a study of their relation to other microorganisms in a complex natural substrate such as the soil.

SOIL ENRICHMENT PROBLEMS

The enrichment of the soil with certain bacteria and fungi in order to stimulate the development in the soil of organisms possessing selective antimicrobial properties has aroused considerable interest among investigators and has had a great appeal to the popular imagination. In spite of the successful results obtained by such procedures (4, 15), the real significance of this stimulation is still open to question. This doubtful attitude to the results obtained is based not necessarily upon experimental facts but rather upon an a priori deduction, namely that the antagonistic organism does not derive any direct nutrition from the antagonized organism, and hence would not be favored in its growth and reproduction by the presence of the latter. The mechanisms of antibiotic action comprise, in a very broad sense, systems that are either enzymatic in nature, ranging from those of lysis of the cells to those of oxidations of certain essential cell constituents, systems that interfere with enzyme or coenzyme reactions, and systems that function in a manner similar to essential metabolites. The fact that specific enzyme mechanisms tend to be favored by the enrichment of the substrate with substances subject to the action of such enzymes gave foundation to the assumption that the development of antagonistic organisms in the soil is favored by enrichment of the soil with the living cells of specific bacteria.

One would hardly question the fact that the isolation (3) of bacteria possessing enzyme mechanisms active against the capsular carbohydrates of bacteria belongs to the above group of reactions. One may even enlarge upon this concept by including mechanisms that catalyze the destruction of the bacterial cell; thus, the isolation of a tyrothricin-producing strain of *B. brevis* (4) was based upon such enrichment processes, although it was later shown (12) that these bacteria are common in the soil and can easily be isolated by much simpler procedures, as by allowing the bacteria to crowd on a plate, that results from the direct plating of heavy soil suspensions upon ordinary nutrient agar media. The case of forced antagonism (11) may further be cited in support of the stimulation theory; certain specific antibiotic properties can apparently be developed or stimulated in an organism, by making it live only in the presence of other organisms; the latter will in time be destroyed by the antagonists. It has also been shown (15) that the addition of living suspensions of bacteria to a soil leads to destruction of the bacteria, and that the rate of disappearance of the added bacteria increases with each repeated addition of living cells; this is accompanied by a corresponding increase in the number of antagonists or organisms possessing the capacity of destroying the added bacteria. A potted unenriched soil gave 15.4 per cent actinomycetes that possessed antagonistic properties; the same soil, after it had been enriched with *E. coli,* gave 42.8 per cent of antagonistic cultures; when enriched with a mixture of different bacteria, as many as 93.3 per cent of the actinomycetes isolated by the plate method possessed highly active antagonistic properties (16).

The available evidence thus permits the conclusion that certain microorganisms have the inherent capacity of inhibiting the growth of or of killing other organisms. Such properties can be stimulated by the addition of sensitive organisms. This effect may either be due to strain selectivity of the antagonist, to the improvement in the culture medium for the development of the latter, or to the stimulation of a latent and specific antibiotic mechanism in the antagonist.

DESIGNATION AND CLASSIFICATION OF ANTIBIOTIC SUBSTANCES

Since antibiotic substances are produced by living systems, they are made available for study and even for practical utilization long before they have been isolated in a pure state and their chemical nature determined. The nomenclature of these substances has not been based, therefore, either upon their chemical properties, or upon the mechanism of their antibiotic action, or even upon their practical utilization, but rather upon their biological origin. Usually, they are designated after the generic or specific name of the organism from which they are derived. Thus, *pyocyanin* is derived from the specific name of the organism *B. pyocyaneus; penicillin* and *notatin* from the generic and specific name of the fungus *P. notatum; fumigatin, citrinin, clavacin* from the corresponding specific names of fungi, namely *P. fumigatus, P. citrinum,* and *A. clavatus; actinomycin, proactinomycin, streptothricin,* and *streptomycin* from recent or older designations of the general or specific groups of actinomycetes. In some cases, the name is selected on the basis of the mode of action of the substance, its chemical properties, or selective bacteriostatic spectrum; thus we have, in the corresponding order, *pyocyanase,* on the assumption that one is dealing here with an enzyme system, *penicillic acid,* and *gramicidin,* the naming of the latter being based upon its capacity to affect largely Gram-positive bacteria.

The above nomenclature is fully justified. Occasionally, however, it has led to a certain amount of confusion, due primarily to two factors already mentioned: first, the same substance or a closely related compound may be produced by different organisms, thus giving rise to the temptation to designate it by a number of different names, depending on its origin; and second, the same organisms may produce more than one substance, thus leading to the confusion that arises from studying a mixture of substances before they have been separated from one another.

Sufficient information has now accumulated to make possible the recognition of several well characterized types of antibiotic

substances. Each of these types represents a single compound or a group of closely related compounds that are formed by one organism growing under different conditions of culture or by several organisms. Once the chemical and biological properties of a new compound have been established, the compound is placed in the proper position as regards type. Further isolations of the same compound should now be coordinated with the established type. In selecting the type compounds of antibiotic substances, the following five major criteria may be considered:

1. The organism that produces the antibiotic substance.
2. The chemical nature of the substance.
3. The selective microbial properties or the bacteriostatic spectrum.
4. The toxicity of the substance to animals and its *in vivo* activity.
5. The mode of action of the substance upon various bacteria.

A brief survey of the more important types of antibiotic substances that are now recognized will suffice to illustrate their natural groupings and to differentiate the type compounds on the basis of the above criteria.

PRODUCTION OF ANTIBIOTIC SUBSTANCES BY FUNGI

Fungi are now known to produce at least eight types of antibiotic compounds (Table III), some of which have been crystallized. They can be briefly summarized as follows:

1. *Penicillin* and *penicillin-like substances* are produced by different strains of *P. notatum* and *P. chrysogenum* and by a variety of other fungi. All the preparations have the same or nearly the same bacteriostatic spectra; they are characterized by low toxicity to animals and by a similar mode of action upon bacteria. Whatever chemical differences may finally be determined between these preparations, they may prove to be only minor in nature. The introduction of several new names for similar preparations obtained from different organisms or from

the same organism under different conditions of culture has been a cause of much confusion. Thus, several penicillin-like substances have been designated as *flavicin, gigantic acid,* and *parasiticin,* and several synonyms have been given to the first of these, namely, *aspergillin, flavatin, flavicidin.* These synonyms and even designations of original preparations obtained from different fungi have little justification.

TABLE III

Antibiotic Substances Produced by Fungi

Substance	Organism	Toxicity to animals*	Bacteriostatic spectrum†
Penicillin	*P. notatum-chrysogenum,* and others	0	++++
Gliotoxin	*Trichoderma, G. fimbriatum, A. fumigatus,* and others	++	+++−
Chaetomin	*Ch. cochliodes*	+	++++
Aspergillic acid	*A. flavus*	+	++
Fumigacin	*A. fumigatus*	+	++
Citrinin	*P. citrinum, Aspergillus* sp.	+	+
Clavacin	*A. clavatus, P. patulum,* and others	+++	++−−
Penicillic acid	*P. puberulum, P. cyclopium,* and others	+	+−

* 0 = nontoxic or very limited toxicity; + = low toxicity; ++ = fairly toxic; +++ = toxic; ++++ = highly toxic.

† Relative activity against specific bacteria on basis of Gram stain.

2. *Gliotoxin* is produced by fungi belonging to the genera *Trichoderma, Gliocladium* (*G. fimbriatum*) and *Aspergillus* (*A. fumigatus*). It contains both nitrogen and sulfur and is formed either alone or in a mixture with other antibiotic substances. It has a much wider antibacterial spectrum than penicillin and is also active against various fungi.

3. *Chaetomin* has so far been isolated only from a single

strain of *Chaetomium* (*Ch. cochliodes*). It contains both nitrogen and sulfur. It is similar in the nature of its spectrum to penicillin, in its solubility to gramicidin, and in its chemical properties to gliotoxin. It differs in many respects from these compounds, especially in its non-activity *in vivo,* since it is readily and completely absorbed by the red blood cells, and is thus inactivated.

4. *Aspergillic acid* is produced by certain strains of *A. flavus.* It is a nitrogenous, but non-sulfur bearing compound, and is selective in its action upon bacteria.

5. *Fumigacin* (*helvolic acid*) is a nitrogen- and sulfur-free substance, also described as helvolic acid. It is produced by *A. fumigatus,* a group of fungi capable of forming, in addition to fumigacin and gliotoxin, two other antibiotic substances, closely related quinones, namely fumigatin and spinulosin.

6. *Citrinin,* also a quinone, is formed by species of *Penicillium* (*P. citrinum*) and *Aspergillus* (*A. candidus*). It has limited activity against Gram-positive bacteria.

7. *Clavacin* is produced by a number of fungi belonging to the genera *Aspergillus* (*A. clavatus*), *Penicillium* (*P. claviforme, P. patulum*) and *Gymnoascus.* This is the reason for the many names given to it, namely *claviformin, clavacin, patulin* and *clavatin.* It is a very common antibiotic substance of the ketone type.

8. *Penicillic acid* is the first antibiotic substance produced by fungi, that has ever been crystallized (1913), although it was not originally recognized as such. It is also a ketone and is similar in many respects to clavacin, although it is not as active. The same may be said of *kojic acid, fumigatin, puberulic acid, puberulonic acid,* and other ketones and quinones formed by a large number of fungi. They have limited bacteriostatic activity against Gram-positive and Gram-negative bacteria.

A group of oxidases, produced by many fungi and active in the presence of glucose or other carbohydrates, may also be added to this list. Their designations, based either on the organism producing them or upon their activity against specific

bacteria, are penatin, *E. coli* factor, notatin, corylophilline, and mycoins.

Other types of antibiotic substances will no doubt be added, in the course of time, to the above list, since comparatively little information is still available concerning the production of such substances by Myxomycetes, Basidiomyces, as well as many members of the Fungi Imperfecti, such as members of the genus *Fusarium.*

PRODUCTION OF ANTIBIOTIC SUBSTANCES BY ACTINOMYCETES

Several well defined type-compounds are now known to be produced by actinomycetes (Table IV):

1. *Actinomycin* is a pigmented, nitrogen-containing ring compound. It is active largely against Gram-positive bacteria and only to a limited extent against Gram-negative organisms.

TABLE IV

Antibiotic Substances Produced by Actinomycetes

Substance	Organism	Toxicity to animals*	Bacteriostatic spectrum†
Actinomycin	*A. antibioticus*	+++++	+++−
Proactinomycin	*Pr. gardneri*	+++	++−
Streptothricin	*A. lavendulae*	++	++−−
Streptomycin	*A. griseus*	+	++−−
Micromonosporin	*Micromonospora* sp.	?	++

* Relative toxicity.

† Relative activity against specific bacteria on basis of Gram stain.

It is highly toxic to animals and is produced by few strains of *Actinomyces antibioticus.* Some strains produce a second, non-pigmented compound designated as actinomycin B, less active than the pigment, originally described as actinomycin A.

2. *Proactinomycin* is a basic compound produced by *Proactinomyces gardneri.* It is soluble in ether and in water and is largely active against Gram-positive bacteria.

3. *Streptothricin* and *streptomycin* represent a group of nitrogenous compounds, basic in nature and active against both Gram-positive and Gram-negative bacteria. They are characterized by a rather limited toxicity to animals. They are produced by two different organisms, namely, *Actinomyces lavendulae* and *Actinomyces griseus,* respectively. The substances themselves differ in their respective bacteriostatic spectra and in the specific toxic effect upon animals. However, they possess certain common properties, such as solubility, mode of action upon bacteria and limited toxicity to animals.

4. *Actinomycetin, micromonosporin,* and *Actinomyces lysozyme* are compounds largely active against Gram-positive bacteria. They are produced by various species of *Actinomyces* and *Micromonospora;* they resemble, in their nature, proteins and, in their action, proteolytic enzymes.

PRODUCTION OF ANTIBIOTIC SUBSTANCES BY BACTERIA

Antibiotic substances are produced both by various non-spore-forming and spore-forming bacteria (Table V). Several

TABLE V

Antibiotic Substances Produced by Bacteria

Substance	Organism	Toxicity to animals	Bacteriostatic spectrum
Pyocyanase	*Ps. aeruginosa*	+	++−
Pyocyanin	*Ps. aeruginosa*	+	++−
Iodinin	*Chromob. iodinum*	?	++
Tyrothricin	*B. brevis*	+	++++
B. simplex factor	*B. simplex*	?	+++*
Subtilin	*B. subtilis*	?	+++

* Highly active against certain fungi.

representative types belonging to each group have already been isolated and studied in detail. These can be briefly summarized as follows:

1. *Pyocyanase* is the first antibiotic substance that has ever been concentrated and described. Although it has been known for more than half a century, it has never occupied a prominent place as a chemotherapeutic agent, chiefly because of the instability of the preparation and the great variability of the bacteria producing it.

2. *Pyocyanin* and its derivative *hemipyocyanin* are two pigmented compounds produced by *Pseudomonas aeruginosa.* They possess relatively limited activity against a variety of bacteria.

3. *Iodinin* is a pigment produced by *Chromobacterium iodinum,* having only limited bacteriostatic action.

Among the spore-forming bacteria, the following type compounds must be recognized:

4. *Gramicidin* and *tyrocidine* (*tyrothricin*) are two polypeptides highly active against Gram-positive bacteria and certain few Gram-negative organisms. They are produced by strains of *Bacillus brevis,* commonly found in the soil.

5. *B. simplex* factor is a substance formed by certain spore-forming bacteria of the *Bacillus simplex* type. It is active against fungi and various bacteria.

6. *B. mycoides* (Much), *B. mesentericus* (Pringshelm), and *B. subtilis* (subtilin) factors. Certain strains of these spore-forming bacteria produce one or more substances that are active against various bacteria.

PRODUCTION OF ANTIBIOTIC SUBSTANCES BY OTHER ORGANISMS

In order to make the above list complete, attention should also be directed to the fact that many other living systems, ranging from viruses to higher green plants and animals, produce substances that are in many respects comparable to the typical antibiotic agents. Some of these may be listed here:

1. *Viruses.* The antagonistic action of one virus upon another is well recognized. The possibility that these effects are in some manner comparable to the action of antibiotic substances is indicated, but until more specific information is obtained, this supposition must be considered as highly hypothetical.

2. *Algae.* The production of chlorellin by a species of *Chlorella* points to the formation of antibacterial agents by at least some members of this group of microorganisms.

3. *Higher plants.* The antibacterial action of the juice of cabbage, onions, and certain other plants has been recognized (6). The designation of *phytoncides* has been given to these substances in Soviet Russia and elsewhere.

4. *Animals.* The ability of protozoa to digest bacteria often involves mechanisms destructive to the latter. Various tissues and excreta of higher animals produce lysozyme (egg white, tears), which is somewhat similar, in its selective action upon bacteria, to the typical antibiotic substances.

MODE OF ACTION OF ANTIBIOTIC SUBSTANCES

The metabolic reactions of different microorganisms vary considerably. The mere fact that some require for their nutrition complex organic substances containing growth-promoting or biotic agents, whereas others can synthesize their cell substance from simple organic and even inorganic salts bears evidence to such existing differences. Their ability or inability to synthesize such biotic agents required for growth, their ability to form specific enzymes, as well as the as yet little understood "bacterial hormones" essential for cell multiplication are other contributing factors. The action of antibiotic substances upon bacteria cannot be disassociated from the possible mechanisms of biotic vs. antibiotic, enzyme and coenzyme vs. antibiotic, or hormone vs. antibiotic systems, or other mechanisms made possible by the complexity of the living cell.

Recent studies of the mechanism of antibacterial action of purely chemical chemotherapeutic agents led to rather definite concepts. This action was believed to consist in depriving the bacteria of the use of enzymes or metabolites by various types of interference. The nutritional requirements of the organisms thus inhibited are more exacting than those of organisms in their normal state. *E. coli* and *S. hemolyticus,* for example, when inhibited by acriflavine components, were found to require

for further growth two types of material not normally needed, one of which could best be replaced by nucleotides, and the other by a concentrate of amino acids, especially phenylalanine (8).

On the basis of the information now available, the concepts of bacteriostasis gained from the study of chemical agents may be enlarged and the following mechanisms of cell inhibition tentatively presented:

1. The antibiotic substance interferes with bacterial cell division, thus preventing further growth of the organism. The cell becomes elongated, reaching abnormal size; unable to divide, it gradually dies.

2. Certain antibiotic agents in bacteriostatic concentrations may have no effect on the metabolic rates of bacteria, though they inhibit cell multiplication. This may result in the production and accumulation of metabolic products injurious to bacterial growth.

3. The antibiotic substance interferes with certain metabolic processes of the microbial cells, by substituting for one of the essential nutrients.

4. The antibiotic substance affects the vitamin utilization of the organism. The staling effect of a medium, frequently spoken of in connection with protozoa as "biological conditioning" of the organism, may serve as an illustration. Other effects could be cited concerning the neutralization of an essential vitamin by an antibiotic substance, a phenomenon often referred to as "antagonism."

5. The substance competes for an enzyme needed by the bacteria to carry out an essential metabolic process.

6. The substance interferes with certain enzymatic systems, such as the respiratory mechanism of the bacterial cell, especially the hydrogenase system, the phosphate uptake by the bacteria accompanying glucose oxidation, as in the action of gramicidin (7), or other enzymatic reactions.

7. The substance directly inhibits cellular oxidations, particularly those involving nitrogenous compounds.

8. The substance acts as an enzyme system and produces, in

the medium, oxidation products, such as peroxides, injurious to the bacterial cell.

9. The substance favors certain lytic mechanisms in the cell, whereby the latter is destroyed; this mechanism may either be primary or secondary in nature.

10. The antibiotic substance may interfere with the sulfhydryl group which is essential for cell multiplication. This was shown (5) to hold true for mercurials and other chemical antiseptics. The possible interrelationship between the sulfhydryl group and true antibiotics has recently been indicated (2).

11. The antibiotic substance acts as a surface-tension depressant, as in the case of gramicidin and tyrocidine (7).

12. Bacteria subjected to the action of an antibiotic substance may develop mechanisms that render them resistant to its action; they may even inactivate the substance. This is true, for example, of penicillinase, the enzyme capable of bringing about the rapid destruction of penicillin.

Because of differences in these mechanisms, some antibiotic substances are primarily bacteriostatic (gramicidin, actinomycin), whereas others are highly bactericidal (tyrocidine, clavacin). The bacteriostatic and bactericidal mechanisms of antibiotic substances are influenced by a number of factors, including the nature of the substance, the nature of the organisms acted upon, the nature of the substrate in which the action takes place, the relative concentration of the substance, the initial numbers of bacteria and the state of their activity, and the temperature and period of incubation.

UTILIZATION OF ANTIBIOTIC SUBSTANCES FOR CHEMOTHERAPEUTIC PURPOSES

Among the several fields of possible application of antibiotic substances, none is of greater significance than their potential utilization for the treatment of infectious diseases of men and animals. Although a number of different substances have already been isolated from fungi, bacteria, and actinomycetes,

only very few have so far found application in the control of disease. This is due to the fact that most of the substances isolated to date have not met the general requisites of chemotherapeutic agents. Thus, many of the antibiotics have proved to be general protoplasmic poisons which not only destroyed the microorganisms inciting the disease but also the host which harbored them. Other substances were found to be insoluble in water and tissue fluids thereby restricting their use to the treatment of local infections. On the other hand, a number of the antibiotics proved to be inactivated by body fluids or inhibited *in vivo* by their fixation to serum proteins or to the other blood constituents. Finally, certain agents were handicapped in their effect by being rapidly excreted from the animal body or by causing a number of undesirable side effects.

Because of the foregoing limitations, only one substance, penicillin, is being utilized at present for the systemic treatment of bacterial infections, although others such as gramicidin and tyrothricin are of value in certain localized infections. A second group of substances including streptomycin, streptothricin and possibly gliotoxin also appear to offer definite promise as chemotherapeutic agents. These substances possess high antibacterial activity and are more or less devoid of the undesirable properties listed above. Penicillin and tyrothricin are active largely against Gram-positive bacteria, and the members of the second group are active against both the Gram-positive and Gram-negative bacteria, as well as against certain pathogenic and saprophytic fungi. Although similar in some respects, the above preparations possess distinct and characteristic antibiotic spectra; they are different in chemical composition, and possibly in their mechanism of action on the invading microorganisms and on the host. They are thus found to represent type compounds for disease control, and they offer promise that other, possibly even more effective, derivatives may be obtained from them when more is learned about their chemical nature.

Several other antibiotic substances, such as pyocyanase and actinomycetin, have also found limited application or their possible use has been indicated.

One may be justified, on the basis of the accumulated information, in indicating the progress so far made in obtaining antibiotic substances for the control of various infectious diseases of man and animal.

Group I. Diseases caused by Gram-positive bacteria and certain Gram-negative cocci. These organisms are among the most sensitive to antibiotic agents, several of which (tyrothricin and penicillin) have already found practical application.

Group II. Diseases caused by Gram-negative bacteria. These organisms are more resistant to most antibiotic agents, several of which, however, are now known to be capable of combating diseases caused by these organisms, and of which at least one (streptomycin) offers promise of success.

Group III. Diseases caused by acid-fast bacteria. Because of the peculiar characteristics of the diseases incited by organisms of this group, they have proved to be among the most resistant to chemotherapy. *Mycobacterium tuberculosis,* despite high sensitivity to many antibiotics *in vitro,* can be attacked in the body only in a manner which involves selective tissue penetration. Fortunately, however, there are already suggestions that such an agent can be found.

Group IV. Virus diseases. No distinct substance has so far been found capable of combating these diseases as a whole. Because of the varied chemical versus biological nature of the disease-producing agents involved, no substance active against all of these agents can even be expected. So far little hope can be extended of isolating substances active upon individual viruses, although the possibility exists that in time such substances may be obtained.

Group V. Rickettsial diseases. Little can yet be said concerning these organisms and the method of treating the diseases involved from an antibiotic point of view. To date very little has been done in this direction.

Group VI. Spirochaetal diseases. Several antibiotic substances (penicillin, streptomycin) are now known to have a remarkable effect upon diseases caused by spirochaetes, and there

is no doubt that they will gradually supplement or even supersede the present treatments.

Group VII. Trypanosomiasis, malarial, and other protozoan diseases. There is some evidence that some antibiotic agents can act upon certain of these. It remains to be determined, however, whether these agents can supersede the arsenical, quinine, and other treatments now in use.

Group VIII. Diseases caused by larger animal forms. Little can be said about these at present.

Group IX. Fungus diseases. A number of antibiotic substances (pyocyanin, gliotoxin, clavacin) are now known to have marked fungistatic and fungicidal properties. Undoubtedly some of these will in time find application in the control of specific diseases caused by fungi.

On the basis of the above observations, even at the risk of appearing to be a false prophet, one cannot help but say that we can look forward to the discovery of new antibiotic agents that will be utilized for combating human scourges for which no effective means of control are at present available.

SUMMARY

The discovery of new chemical agents, known as antibiotic substances, whereby one living system is capable of inhibiting the growth of another or of actually destroying the other system has opened to the biologist new vistas for the study of ecological relationships among microorganisms. The fact that these phenomena involve the action of saprophytic organisms upon disease producing bacteria and fungi has added to the great potentialities thus uncovered; for the biologist—a better understanding of the physiological reactions in the microbial cell; for the chemist—new chemical mechanisms for destroying disease-producing agents; for the physician, the veterinarian and possibly the plant pathologist—new actual or potential chemotherapeutic agents for combating human, animal, and plant diseases.

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NEW DIRECTIONS IN RENAL MORPHOLOGY: A METHOD, ITS RESULTS AND ITS FUTURE[1]

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TO the privilege that an invitation to address your Society brings is added a considerable responsibility if one is to talk of the structure and function of the kidney. This I have come to appreciate in looking through the list of lecturers who have preceded me, for there are found the names of those around whose work has grown our present-day knowledge of how the kidney is built and how it functions. The anatomists, Huber and Huntington; the experimentalists, Pearce, Brody, McNider and Richards; the clinicians, Christian, Janeway and Addis; the pathologists, Aschoff and Goldblatt, and, most recently, the physiologist, Homer Smith—you can imagine, I think, the hesitancy of one who is asked to follow where these men have led and to carry on in their tradition.

There is one lecture in this series, given in 1909 by Professor Huber (1), "On the Morphology and Structure of the Renal Tubule," to which I would refer in particular. It would indeed be gratifying if there were present tonight some one who had heard him speak, for what I shall say may be considered almost a direct sequel to his work, both in method and content. For Doctor Huber, along with Peter in Greifswald (2) was one of the few morphologists who viewed the renal unit in what Trilby literally meant by "the all together," the simple statement, you will remember, of the obvious truth that to observe the whole, one must see its parts in continuity and normal relation.

These two men, Peter and Huber, solved the problem of continuity in their work by the method of maceration and dissection. What I shall describe is an extension of this method in the field of both the normal and abnormal. And since I wish to show

[1] Lecture delivered December 21, 1944.

the new directions that research in renal morphology can take with such a method rather than the detailed results of its application, I shall give only very general descriptions of where its use has led, in the hope that this will indicate how it may be used in further examination, and at times to a reasonable solution, of various questions of renal morphology and function.

THE GROSS MORPHOLOGY OF THE ABNORMAL NEPHRON

Huber and Peter having established the morphology of the normal nephron, a pathologist would, I think, turn most naturally to the chronic forms of Bright's disease for examples of the abnormalities that may develop under abnormal conditions. I only shall refer in passing to this, the first step, in our work, for its results have been fully reported (3). Infinite diversity perhaps sums up the myriad forms that the nephron may take under the stress of adverse circumstance, regressive change alternating with progressive, not only in neighboring nephrons but in the same nephron, and indeed within one morphological division of a single unit, such as the proximal convolution. One generality at least may be stated, that no particular alteration is characteristic of any particular form of Bright's disease.

When the arrangement of these abnormal nephrons in the "contracted kidney" is examined, two architectural end effects are seen. The kidneys from individuals in which the course of the disease has been continuous are of relatively uniform construction and show throughout simple regressive organ alteration (metallaxis), while in kidneys where remissions have broken the inexorable approach of renal failure, the anatomical evidences of reparative and progressive tissue response are found. The architectural pattern in the two instances is therefore quite different, but, as in the deformed individual nephron, no form of altered kidney is peculiar to any type of Bright's disease. By whatever course the end has been reached, the organ structure of contracted kidneys is similar. The clinician therefore need have no feeling of frustration if at the end he cannot differentiate one from the other by functional means.

THE APPLICATION OF THE GROSS MORPHOLOGICAL FINDINGS TO FUNCTIONAL PROBLEMS

Gross anatomical data, though not in themselves without attraction to a certain type of mind, are generally admitted to gain greatly in importance if dynamic and living implications can be read into their dead immobility. In this worthy purpose, the dissected nephrons lend themselves well to the examination of experimental procedures, since the nephron as a unit and not, as in the histological section, a thin segment of its glomerulus or tubule is examined. For example in vital staining of the kidney with such dyes as trypan blue only an examination of them by this means can afford a demonstration of how these substances are handled by the normal nephron acting as a complete functioning unit or show what alterations occur in the functional mechanisms of the abnormal nephron (4, 5). It was found, as Suzuki (6) had concluded from his study of sections, that the dye was selectively absorbed and stored only in the proximal convolution of the normal nephron and in decreasing concentration as one departs from the glomerulus. Our study of abnormal nephrons from the kidneys of vitally stained dogs suffering from chronic canine Bright's disease revealed, however, marked differences in the way these nephrons handle the dye. One difference was purely quantitative, for the large hypertrophied nephrons absorbed and stored the dye in a normal manner but in greatly increased amount. In this observation we find at least an indirect and partial answer to the frequent question as to whether the hypertrophied nephrons of chronic Bright's disease are not only bigger but really better. Nephrons of abnormal structure, with atrophied and atypical epithelium, showed on the other hand qualitative changes in their functional behavior, since they in part did not absorb and store the dye and under other circumstances allowed it to diffuse into the tubule wall.

The substances concerned in these experiments and the mechanisms involved are not those of normal physiological activity, but the observation of their derangement raises the question as to whether the methods of handling other equally foreign materials,

such as are used in "clearance" measurements, may not also be altered, and, consequently, if the application of these clinical tests to the abnormal organ can be interpreted with the same significance as when applied to the normal organ.

When this criticism was first made (5), the only comment deemed necessary by one reviewer was that it "stems largely from an improper understanding" (7, p. 307). A later examination of the question by other investigators, however, and this based on experiment rather than dialectic, has led them to the conclusion that the "use of diodrast plasma clearance as a measure of renal plasma flow and of inulin clearance as a measure of glomerular filtration is not justified in the uranium damaged kidney" (8, p. 160). If this be true in the relatively simple derangements of the uranium lesion, how are we to interpret them in the structural and functional complexities of a chronic glomerular nephritis? In answering this question either the experimental or the ratiocinatory approach (9, 10) may be used. In awaiting the former, the morphologist can at least feel that his criticism was based on something more than his admittedly limited comprehension of current functional theories, which have indeed "taken conceptual wings."

Another application of the method of dissection to the furthering of functional study was the contribution of its advantages of continuity to Richards' method of study of renal function (11). In the living amphibian kidney the nephrons may be seen and easily identified as they lie under the microscope during the removal of glomerular or tubular fluid from the functioning nephron. In the mammalian kidney all that can be seen is a congeries of tubules, so that although the experimenter can place his pipette within a tubule, he has no means of knowing from where his sample has been obtained. It was possible, however, for us to dissect out the functioning mammalian nephrons that Doctor Walker had tapped, to identify the point where the pipette had pierced the wall of glomerulus or tubule and to measure its place in the nephron as a whole. By this means the absorption of sugar in the proximal convolution in the normal and phloridzinized ani-

mal was accurately followed and other determinations made of changes occurring in the glomerular filtrate during its passage down the tubule.

To this point, I have considered work which has been completed in the sense that its data have appeared in published form, but from now on I shall be describing results of unpublished work in progress.

It would seem unnecessary to state that growth, the ever-visible correlation of function and structure in nature, can only be adequately examined by a method which measures the whole of the organ under investigation. Though a vast amount of work has been done in the past on growth of the kidney in relation to the work done by it, especially in the case of the so-called "compensatory hypertrophy," yet in no case has this fundamental requirement been met. We find instead, either that the sum total of many organs (nephrons) has been estimated by weighing the growing kidney or increase in a single dimension of some part is taken as an index of its growth; for example, the cross-sections of tubules have been measured and no consideration given to their length! During our earlier work when large nephrons were first seen complete (3), it became quite evident, that what had been suspected by the use of less adequate technique (12) was a fact, namely that all parts of the nephron do not respond similarly by growth to the stimulus of an added functional burden. Let me illustrate in a quantitative manner the complex relations that appear when the method of examination reveals the whole of the picture.

In Figure 1 are shown camera lucida outline tracings of typical dissected nephrons from rats whose kidneys were given me by Thomas Addis. These animals had been the subjects of an experiment devised by him to test the physiological effect of an added functional burden on the growth of the kidney. This was accomplished both by decreasing the amount of renal tissue that handled a constant load (compensatory hypertrophy) and by adding to the metabolic burden by increasing the protein content of the diet.

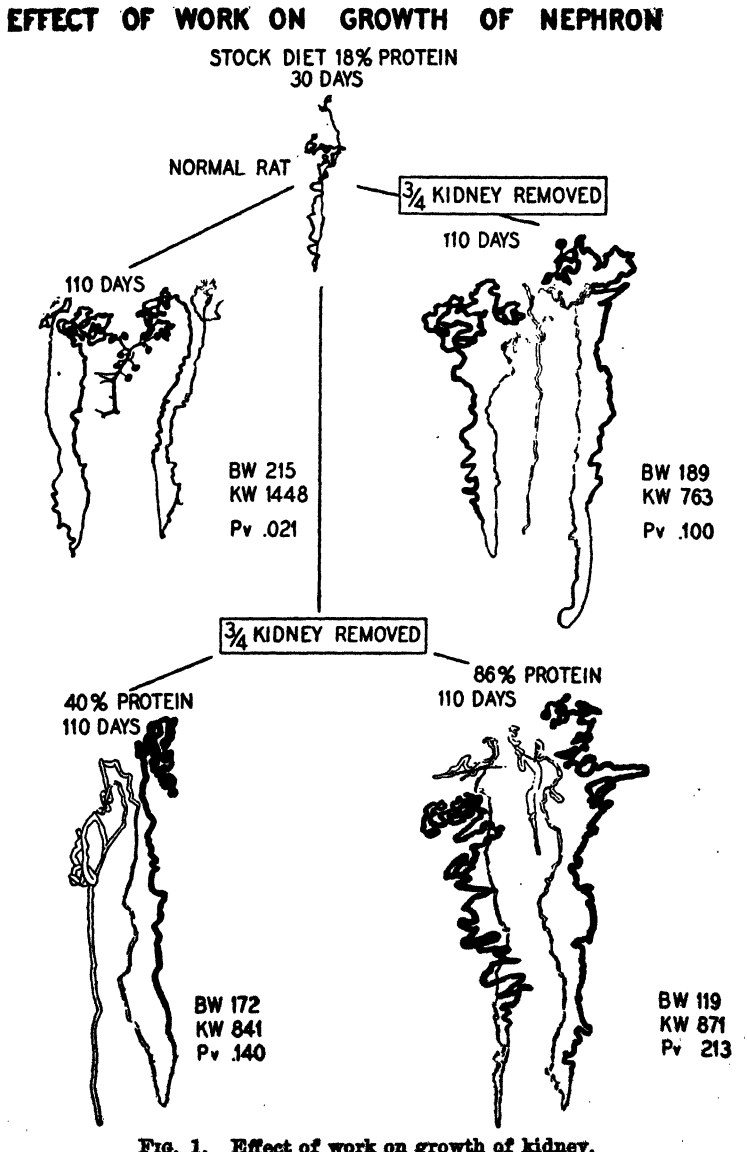

Fig. 1. Effect of work on growth of kidney.

At the top of the figure with its proximal convolution in black is seen a nephron at the beginning of the experiment from a 30-day-old rat on an adequate stock diet containing 18 per cent protein. To the left, nephrons of a representative animal at 110 days are shown. Body weight had increased from 44 to 215 gm., the kidney weight from 400 to 1448 mg. and the volume of the proximal convolution, as calculated from its cross-section and length, from 0.007 cu. mm. to 0.021 cu. mm. To the right are shown typical nephrons of animals of the same age, but from which three-fourths of the kidney substance had been removed at 30 days. These animals may still be considered normal from the standpoint of their renal activity in the sense that their blood is still being cleared to a degree compatible with survival, but the remnant of their kidneys is doing the maximum that normal kidney tissue in this amount can do, for a further reduction results in renal failure. It is apparent in the figure that the nephrons in this overburdened remnant have grown much more than under normal conditions, and that their growth is not a simple over-all and proportionate increase in size of their parts but that the proximal convolutions have become disproportionately large. The volume of this part of the nephron has become about five times that of a normal convolution at this age, though the total kidney tissue, expressed by weight, has become only somewhat more than one-half of that of the normal. Clearly the increased weight of the kidney at the end of the experiment is not, therefore, a proper measure of the growth that occurred in the organ.

It is when the growth of the renal tissue in this experiment is considered as a correlation between structure (size) and function (maintained blood clearance) that the discrepancy between weight (mass) of "kidney" and functional load contrasts most strikingly with the agreement observed between mass of the proximal convolution and functional work done. After three-fourths nephrectomy the "kidney" mass had only doubled from one-fourth and so restored only one-half of the original normal ratio between structural size and functional work done. The mass of one-fourth the original number of proximal convolutions

had, however, increased five-fold, which in view of the difficulties of measurement may be taken as an approximate demonstration of the restoration of the original structural-functional constant.

As a matter of fact the morphologist had no need to feel disturbed as some have been (13) when during the course of the experiment he was faced with the discrepancy between "kidney" weight and functional output, for he could feel certain, since growth could have no meaning unless it is correlation of size and function, that he must be comparing the wrong things. The trick in the experiment came in discovering the equivalent entities, and these became obvious when the method used disclosed the functioning nephron as a complete unit. It could then be seen that its important functioning part, the proximal convolution was performing at the end of the experiment neither more nor less per gram of functioning tissue than it did under the original normal conditions.

The proximal convolutions had therefore grown enough to restore the original structural-functional equilibrium and then stopped, although their capacity for further growth had not been exhausted. This is shown by the experiment of adding still more functional burden to the reduced number of nephrons. In the lower half of the figure are shown the results of increasing the protein content of the diet. The weight of the kidney increases, still inadequately to restore its original normal mass relation to body weight, i.e., functional metabolic load, but the proximal convolution grows to seven and then to ten times its usual size. Unfortunately this is either not enough or what is more likely it is an inefficient or "abnormal" increase in size, for the kidneys of these animals fail to adequately maintain the physiological metabolic levels in the blood and renal failure develops. Associated with functional failure structural abnormalities develop with plugging and dilatation of the tubules so that in the final complexity of the abnormal no analysis of exact relations is at present possible.

It is an interesting fact, that though one can find innumerable studies on the growth of the kidney under abnormal circum-

stances, we have practically no information, save for weight-growth curves, as to how it grows under normal conditions. Since in post-natal life, except for its first period, the number of nephrons is fixed, it is clear that only a knowledge of how the individual unit grows is of value. Peter (2) has given a few random measurements of the growth of the human nephron at different ages, but no proper statistical treatment of the problem exists. During the last year we have been trying to fill this hiatus and it has become progressively and I might add more painfully clear to us why it exists. For since not all nephrons start their growth at the same time and do not grow in the early period of life at the same rate, it becomes necessary to examine the various parts of each nephron in relation to itself. One cannot accumulate a great number of proximal convolutions, distal convolutions or glomeruli and then measure them "en gros." The relation of each division must be compared to the other divisions of the same individual unit, which means that whole and complete units must be dissected out before any measurements are made. This we have done, measuring the diameters and lengths of glomerulus, proximal convolution, Henle's loop and the distal convolution. Since the mass of a functional unit would seem to be the most valuable structural datum for correlation with functional activity, the volumes of the various portions have been calculated. I cannot take time and space to give the statistical analysis of this material, but must ask you to accept the results presented as adequately controlled in that regard.

The growth of an organ is commonly compared to many other varying quantities, such as in our problem, the volume of the different parts of the nephron to time, body weight, body surface or kidney weight. All this has been done with results of varying interest, but I shall mention here only certain examples of relations that bear on the functional aspect of the structure problem. For example, the rate of increase in no single part of the nephron, or for that matter in the sum of the growth increments of its parts, bears any apparently useful relation to increase in weight of the kidney, though certain comprehensible relations do begin

to appear when body weight is used as a basis for comparison. This one might expect from the findings of the previous experiment on growth under functional overloading, and from the fact that other elements beside the nephrons, such as blood vessels and framework grow in both a positive and, in the case of the interstitial tissue, negative sense.

The greatest degree of useful correlation appears however when the growth of individual parts of the nephron is compared to the

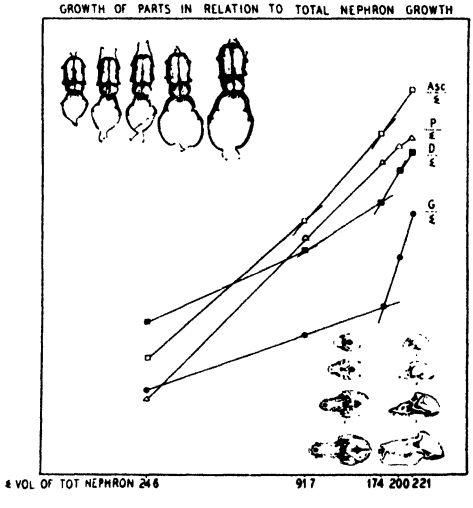

FIG. 2. Heterogonous growth of parts in relation to total nephron growth.

growth of the sum of its parts and here the orderly relation appears not with all parts of the nephron, but with certain functioning parts; namely the proximal convoluted tubule and the glomerulus. Moreover, the relation is not arithmetical or geometric, but shows itself to be exponential. It is such growth increment that Huxley (14) has termed heterogonous growth, its characteristics being that plotting its data logarithmetrically produces a straight line. It will be seen in Figure 2 that when the volume of the proximal convolution is compared logarithmetrically to the volume of the complete nephron an almost perfect

approximation to a straight line is produced. The same is true of the volume of the glomerulus, except that at 70 days, after onset of puberty, the slope of the line is abruptly shifted. A rapid increase in rate of growth produces at this point, in Huxley's terms, an increase in the heterogonous constant of growth, but progress beyond this point is again in a straight line. The ascending limb and distal convolution on the other hand show a change in rate at each point of the curve and are characterized therefore by no growth constant of this sort.

Heterogonous growth is a commonly observed biological relation and in most instances it is an excessively functioning part of the whole which demonstrates its characteristics most perfectly. In Figure 2, examples taken from Huxley's monograph are shown where, from beetles to baboons, heterogonous growth is evident in hyperactive claws and jaws.

The common desire of both morphologist and physiologist is, I presume, a meeting of minds on the common ground of correlation of structure and function. In the good intent of this ideal, certain definitions and limitations are apt to be lost sight of, and a fundamental one illustrating the inherent pitfalls in the problem of correlation is made manifest by the examinations of the renal tissue growth that I have described. Although it may appear at first hand to have only a theoretical semantic interest, in fact such distinctions are of great practical importance, since much time may be lost both in talk and work unless they are appreciated. The gross anatomical and physiological term "kidney," for example, has no rational usefulness in a problem of correlation of structure and function unless it is applied strictly to a homogenous packet of equivalent nephrons, so long as the functional work of the organ is expressed, as it always is at present, by the total output of the organ. Even a normal "kidney" increasing in mass by normal physiological growth does not fulfill this requirement, for changes occur in the relative proportions of its functioning parts and as we have seen the mass of the "kidney" does not correlate with work done by it. On the other hand, certain types of abnormal "kidney" such as acute damage follow-

ing the injection of a toxic agent, may by their uniform damage to all nephrons fulfill the definition of the problem. But in the abnormal "kidney" as seen in chronic Bright's disease of any sort, where every nephron differs from its neighbor in both structure and function no correlation can be expected when work or output is measured as the single algebraic sum of several million variables.

THE CYTOLOGY OF THE NEPHRON

Whatever the valued attributes of gross morphology, cytological detail brings us at least closer to that border, perhaps never to be reached, where structure and function operate in the living process. There has been so far in the literature no description of stained isolated nephrons beyond a casual statement by Huber, that his attempts to stain them had led to no worthwhile results. We have devised a method of staining the dissected nephron which notwithstanding certain difficulties reveals a wealth of cellular detail. In spite of the previous treatment with strong HCl, just as the tensile strength of the tubule is maintained and dissection made possible, so, except for the chromatin of the nuclei, the tinctorial properties of the cells persist to a surprising degree. The method in summary is a modified iron hematoxylin procedure, using ferric chloride as a mordant. With some delicacy of manipulation the dissected complete nephron may be gotten intact onto a slide and there stained and differentiated beneath the cover glass. Its substance is so fragile however that it cannot be cleared in the usual media since shrinkage occurs with the removal of its water content. When examined, the specimen, consisting of the entire organ, is thick as compared with the histological section, but nevertheless appearances are noted that are missed entirely in section and continuity of structure is of course maintained.

Little in detail can be said at this time of the cytology of the normal nephron. I unfortunately cannot show you the complete picture of the constantly shifting pattern as one passes from one division of the tubule to the next, since the size of plate required to show a tubule, say 1 cm. in length, at a magnification adequate

to show detail, perhaps 150 times, would be available only in the Sunday rotogravures of the past. The gradual progression of infinitesimal change in the mitochondrial apparatus of the proximal convolution for example, first suspected by Suzuki (6), and which is duplicated in no other part of the tubule of the nephron, is something which must be seen if its exquisite gradation is to be appreciated. It is gratifying indeed that the one method should thus reveal both the perfectness of this decreasing structural gradient and, as in the experiments with Walker (11), the analogous similarly gradual functional decrease in absorption.

Certain elements of the normal nephron which are not apparent in sections become clearly visible in the complete stained nephron. The diverticula so common in the distal convolution and so very rarely found on any other part of the tubule, are shown in Figure 3. They seem to bear no relation to any abnormality of the renal structures though they do increase with age. For the pathologists, I would add that diverticulitis has not been observed. It also becomes apparent in dissected vessels that the

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FIG. 3. Diverticula on distal convolution from a normal human kidney. Their common occurrence is indicated by their chance appearance in Figures 14 and 28. Magnification 200 ×.

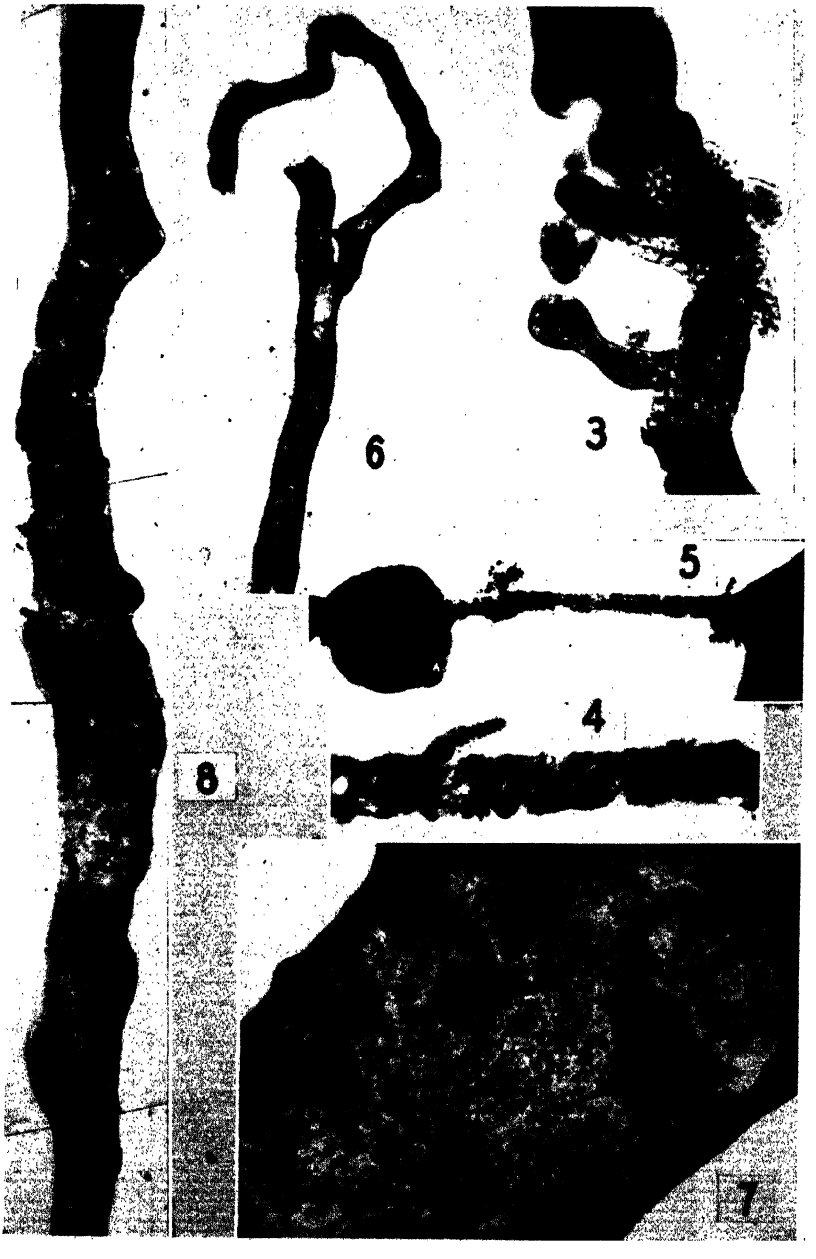
FIG. 4. A terminal branch of an interlobular artery from a human kidney showing spiral arrangement of the muscle bundles. Magnification 200 ×.

FIG. 5. A glomerulus with an afferent arising from the interlobular artery of elephant kidney showing spiral muscle bundles in the former. Magnification 57 ×.

FIG. 6. A connecting tubule joining a peripheral collecting tubule from kidney of rat. Against the light background of the epithelial cells are seen the dark intercalated cells. Magnification 90 ×.

FIG. 7. Higher magnification of a peripheral collecting tubule showing the detail of the intercalated cells. Note the dark granules which fill their protoplasm. Cf. also Figure 32. Magnification 1200 ×.

FIG. 8. Junction of a distal convolution and a connecting tubule from rat's kidney. Above is seen the regular pattern of the densely granular epithelium of the distal convolution; below the scattering of the granular intercalated cells along the clear wall of the connecting tubule. Magnification 500 ×.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muscle fibers in both the interlobular artery (Fig. 4) and in the afferent arteriole (Fig. 5) are arranged in the spiral bundles of a helix. The functional implications of a helical contracting system, not only in the renal arteries, but generally in the systemic arterial system, have at times in the past been discussed as theory, but that such an arrangement has been objectively demonstrated in our laboratory as fact by Strong (15) does not seem to have become generally recognized.

And finally what might be called the lost tribe of renal cells are again disclosed dispersed along the connecting and upper collecting tubules. Described in the 80's as deeply staining objects of inexplicable origin and function and then apparently forgotten, these dark granular cells scattered on the background of the clear cells of the peripheral collecting system stand out in a striking pattern in the stained tubules (Fig. 6). On higher magnification the term "intercalated" appears peculiarly appropriate, as they clearly lie between and are compressed by the predominate cell of the tubule (Fig. 7). Their dark color is due to the presence of many coarse granules that fill their protoplasm and a clue as to their nature and origin appears, thanks to the continuity of the specimen. In Figure 8 is shown the transition between distal convolution above to connecting tubule below. The former is composed of evenly arranged cells filled with the mitochondrial rodlets peculiar to that portion of the tubule, and from this orderly arrangement there is quite literally a scattering of isolated granular cells along the connecting tubule. Remembering that in the embryo it is at this point that fusion of the nephrogenic tubule with outgrowths of the collecting ducts occurs, it seems reasonable to suppose that the intercalated cells of the adult kidney are embryonically misplaced members of the heavily granular epithelium of the nephron tubule. This conclusion is strengthened by the fact that they do not occur throughout the entire collecting system, but are limited to the neighborhood of its connection with the nephrons and also by certain functional similarities between these cells and the epithelium of the distal convolution to which we later shall refer.

Another structure of the nephron assumes a new and more tangible aspect in the stained dissections. This is that collection of cells of unusual appearance on the afferent, and at times efferent, arterioles to which has been given the name of "polar cushion," a modest and not inappropriately descriptive term. To others, it forms one part of an ideal, to the present time purely imaginative "juxta-glomerular apparatus of Goormaghtigh." The other element of this "apparatus" is the macula densa, a patch of cellular irregularity in the epithelium of the distal convolution at its point of attachment to the glomerulus, and the combination has been described as the regulator of glomerular blood flow, operating automatically by changes, not defined, occurring in the fluid of the distal convolution. Not only does it thus become the chief regulator of renal activity, but the occasional presence of granules in its "leiomyo-epithelioid" cells suggests to some that, as an endocrine organ, it may be the source of "renin" and the regulator of the systemic blood pressure as well.

Such is perhaps the most elaborate form that a statement of our knowledge of these structures takes. So far as I know, no one has ever stated the case in its contrastingly simplest form. Over and against the description of the "apparatus" as it exists in the realm of pure reason one might then set the picture seen in the harsher lineaments of structural actuality. Unfortunately, that picture, as it is derived from the study of histological section alone, cannot be clearly drawn, for I think it is fair to state that there is quite general disagreement on almost every point. The cushions on the afferent arterioles are often at best a vague scattering of nondescript, a term I prefer to "leiomyo-epithelioid," cells and as a rule there are no granules to be seen. It is generally admitted that the frequency, size and cellular content of the cushions vary with species, age and even in different parts of the same kidney. The only functional correlations that have been attempted, those of size of cushion and granularity of cells with a state of hypertension, have proven dubious. In examining a section of any kidney, one seems always to find more epithelial macula densa than arteriolar cushions, a discrepancy which is not

easily reconciled with the concept that two elements are reciprocating halves of a single apparatus.

From all that is factually known, therefore, both cushions and maculae might be regarded simply as structural modifications, the result of mechanical stresses, in the one case acting on the endothelial and muscular elements of a thin-walled arteriole which from its location is under peculiar functional strain, and in the other acting mechanically through the fibrous attachment of glomerulus to tubule. The presence of granular cells is, I will admit, not accounted for by this statement of the case, but I prefer to leave one structural stone as yet unused rather than for the sake of architectural elaboration erect such a baroque facade of speculation and hypothesis. And so, as Homer Smith suggested in his lecture to your Society in 1939, "it would seem that the normal kidney could be profitably re-examined." I would add, that a new method of examination might be tried.

In the first instance the method of dissection makes possible a proper statistical examination of the problem, for afferent arterioles in great number may be dissected from a kidney and the presence or absence of the cushions easily noted. We have as yet made no such formal study, but casual observation has made it evident that there is little consistency in the occurrence of them in the kidney of the normal animal of any species, for a spray of a dozen glomeruli and their afferents from a single inter-lobular artery may show only an occasional cushion. If the nephron depends on this element of the "apparatus" for the regulation of its activity, many are therefore uncontrolled.

FIG. 9. An afferent arteriole and glomerulus from the kidney of a cat showing lateral view of the large projecting polar cushion. Magnification 200 ×.

FIG. 10. Edge-on view of the same specimen. Magnification 200 ×.

FIG. 11. Afferent and efferent arterioles and glomerulus from kidney of the dog. Note the large nucleus with nucleolus in a "myoepithelioid" cell on the upper margin of the spindle-shaped polar cushion. Magnification 200 ×.

FIG. 12. A polar cushion on an afferent arteriole from the kidney of a rabbit. The large peculiar cells of the cushion are well shown. Magnification 450 ×.

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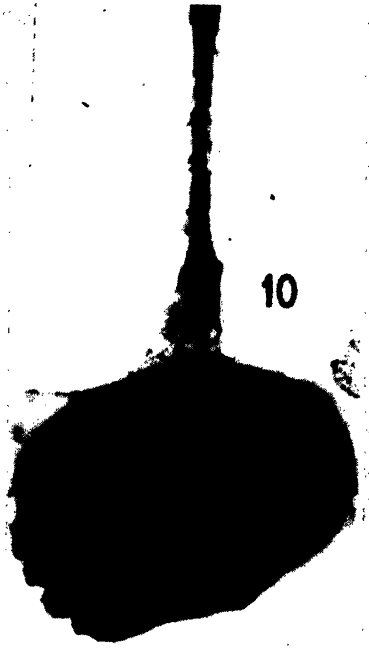
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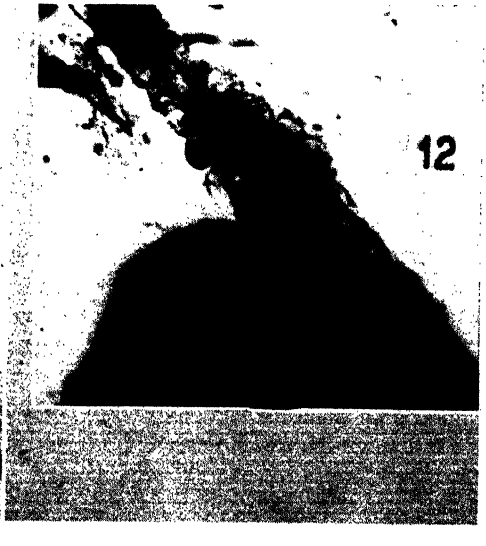
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On the other hand, the dissected material makes clearly evident what was never apparent in the histological section, namely that the cushion is not a tissue, but at least in the structural sense, a definite organ in its own right, the shape, size and topographical relations of which are peculiar to each species. In Figures 9 and 10 are seen in profile and edge-on, the large, flat, spur-like mass of the cushion of the cat. In the dog (Fig. 11) a rounded spindle-like form of somewhat lesser size is present; in the rabbit (Fig. 12) the cellular mass is less compact and regularly contoured and still smaller. In all the afferent vessel passes through the tissue mass excentrically. The pig, rat, guinea pig and man all show these tissues arranged in definite organ architecture, and one could in many instances at least, identify the species by the appearance of the cushion alone.

Due to the thickness of the specimen, the cytological elements of these structures are less clearly seen than in histological section and though the morphological peculiarities of the strange cells that compose them may be distinguished (Figs. 11, 12), nothing new of interest is disclosed by the method. For the same reason the macula densa, obscured in the depths of the tubule wall has not been examined, though I am sure that special means would make it visible and its topographical relations to the cushion might then be more accurately determined.

CYTOLOGICAL LOCALIZATIONS OF METABOLIC ACTIVITY IN THE NEPHRON

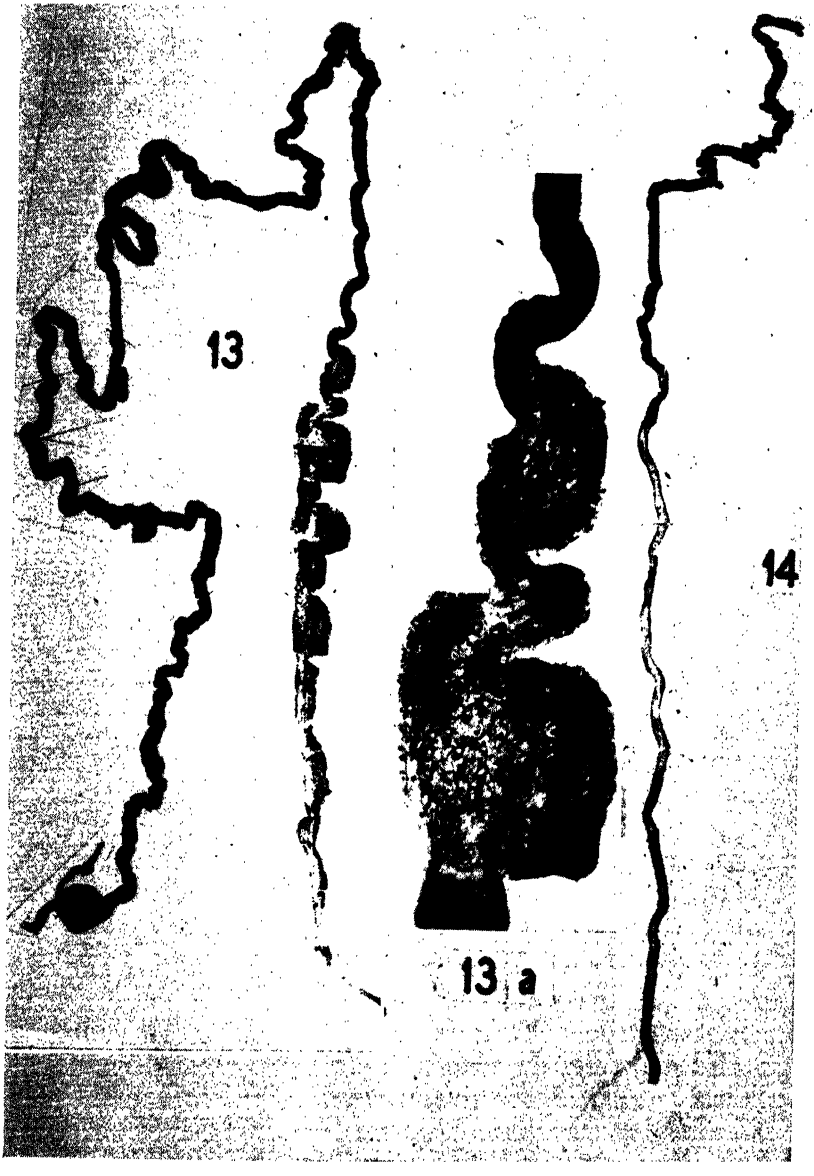
Localization of various metabolic processes in certain parts of the tubule is a concept that has received much support both from functional and histological investigation. Again it is self-evident that completeness of morphological data in this problem can be only obtained when the cellular detail of the entire nephron is examined as a unit in its natural continuity. Since we are now able to study the cytological characteristics of complete specimens this fundamental requirement has been met and the following summary of work in progress will show the extent to which the method has been used and indicate the possible extension of its advantages to other problems of similar nature.

ALTERATIONS IN CARBOHYDRATE METABOLISM

As I have previously shown you, the method of dissection has made possible the definitive observation by functional experimentation that sugar is absorbed normally in the first half of the proximal convolution of the mammalian nephron. The localization of carbohydrates by observation of structural change in the dissected nephron is limited however to an examination of those substances that leave evidence of their accumulation within the renal cells by the production of intracellular spaces. Such spaces, formerly filled with glycogen, are found in the tubular epithelium in diabetes. That the huge foam-like Armanni-Ehrlich cells distend the swollen tubule is plainly evident in histological section, but the decision as to what part of the nephron is involved is more difficult. According to most authorities these cells are limited to the ascending limb of Henle's loop, to others they are found only in the proximal convolution and to some they are present in both.

It is a simple matter to dissect out complete nephrons from the kidneys of human diabetics and in the stained specimens it is readily seen (Figs. 13, 13a) that the terminal part of the proximal convolution is the only portion in which distension and vacuolization of the cells occurs. The degree and extent of the resulting deformation of the nephron is also much more apparent than it is in the histological section.

It must be clearly understood that the appearances just described are not to be taken as the complete picture of the glycogen content of the nephron. As a glance at a section stained specifically for glycogen makes evident, a more moderate and finely divided increase in this substance producing no vacuolization of the cells occurs generally not only throughout the nephron but in other tissues and organs as well. This diffuse deposit is not visible in the heavily stained dissected nephrons though it may be suspected by the more lightly staining cytoplasm of the cells in certain regions. Such a region is found in the terminal broad portion of the ascending limb just below its junction with the distal convolution (Fig. 14). To summarize then, in human dia-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betes glycogen is deposited in greatest amount in the terminal proximal convolution and only here is deformity of cells and tubules produced, though there is evidence that a very much lesser increase may produce barely visible protoplasmic disturbances in the terminal segment of the ascending limb.

An examination of animals (rats, cats and dogs) made diabetic by pancreatectomy shows an interesting species variation in the handling of the carbohydrate. I am endebted to Doctors Eaton McKay of La Jolla and F. D. W. Lukens of the University of Pennsylvania for this material. In the first place, it is difficult to produce glycogen increases in the kidney sufficient to be visible as vacuoles in the renal epithelium even when chemical examination shows a considerable increase in the renal tissues. When the accumulation of the typical Armanni-Ehrlich cells is produced, however, it is always in the ascending limb and only in that terminal segment adjacent to the distal convolution where evidences of slight deposition were observed in the human nephron. In no

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FIG. 13. Complete glomerulus with afferent and efferent arterioles and proximal convolution from the kidney of a human diabetic. The specimen is over-stained to bring out contrasts between those cells which contain no excess of glycogen and which are densely black and the clear Armanni-Ehrlich cells which are distended with vacuolar spaces which contained glycogen. The latter are found only in the terminal segment of the proximal convolution. Magnification 26 ×.

FIG. 13a. Detail from same preparation of a distorted swelling on the terminal segment of the proximal convolution showing the mosaic pattern of Armanni-Ehrlich cells. Magnification 100 ×.

FIG. 14. The ascending limb and distal convolution, with diverticula, of the same nephron as Figure 13. There is no visible glycogen in the densely stained distal convolution or in the more slightly granular cells of the lower part of the ascending limb. In the upper portion of the latter just before its passage into the distal convolution there is, however, a considerable infiltration with glycogen as evidenced by the increased clarity of the cells. There is no deformity of the tubule and no production of the Armanni-Ehrlich type of cell. In the dog, cat and rat the localization of these changes is the converse, Armanni-Ehrlich cells occurring only in the upper ascending limb, never in the proximal convolution. Magnification 26 ×.

case has accumulation in the proximal convolution, if present, been sufficient to be visible.

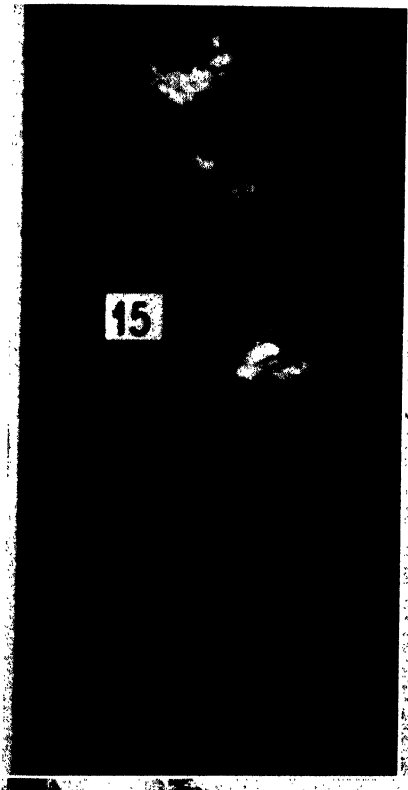
THE METABOLISM OF FATS

It has long been known that in certain species fat in visible form appears normally in the tubule cells of a certain limited part of the nephron. In the cat this deposit is in the upper half or two-thirds of the proximal convolution, the medullary portion remaining free. In the dog the converse is true. In the cat, then, one might suppose that the distribution was similar to that of the vital dye previously described, which, beginning in greatest concentration at the glomerulus gradually decreases. An examination of a complete proximal convolution of a cat shows that this is not so. The fat is in equal concentration from its origin until the end of the infiltrated portion is reached and there it abruptly ends. There is nothing therefore in the distribution of the droplets to suggest that the fat has been absorbed, as was the dye, from fluid passing down the tubule lumen, but rather there is the appearance as if it had accumulated there because of some peculiar localized metabolic condition within the cells of this portion of the convolution. This supposition is strengthened by the converse limitation of the fat in the dog's convolution to its terminal segment.

FIG. 15. A complete nephron from a human kidney of lipoid nephrosis. The greatly swollen proximal convolution is seen to be irregularly infiltrated with brightly refractile fat. The normal ascending limb and distal convolution which contain no excess of fat lie to the right. Magnification 16 ×.

FIG. 15a. Detail of a group of tortuous coils in the terminal segment of the proximal convolution, a distortion which has been produced by the intense deposit of fat in the epithelial cells. Magnification 48 ×.

FIG. 16. Proximal convolutions from the same kidney stained with cadmium-Sudan III. In the upper specimen all cellular detail is obscured by an intense infiltration of fat in the form of fine fat droplets. In the lower specimen the deposit of dark stained fat is more patchy and so intense as to cause bulging of the swollen cells through the membrana propria. Magnification 100 ×.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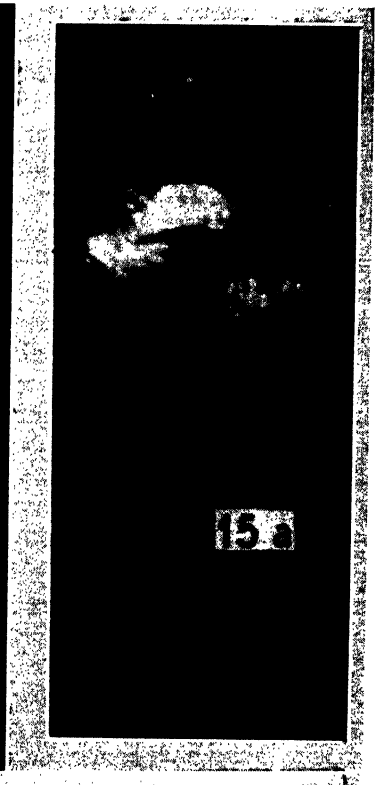
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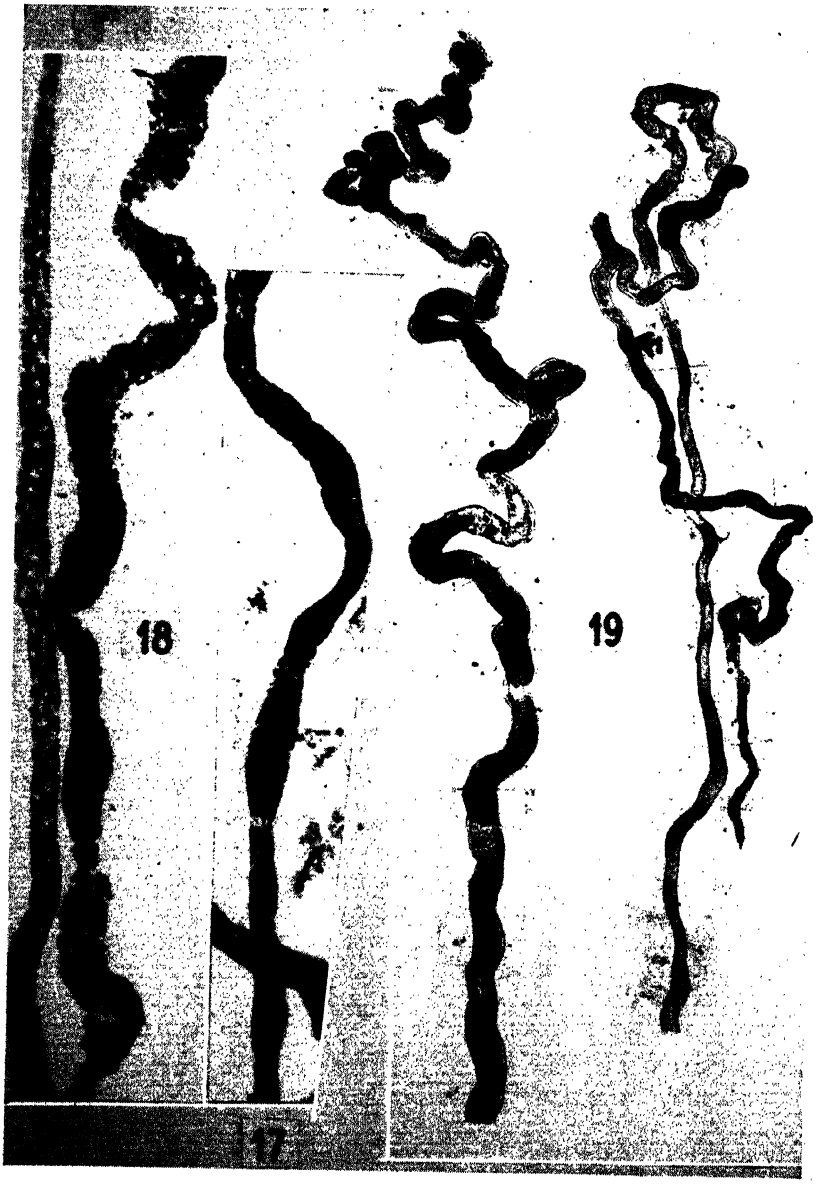
Since Richards' demonstration of the importance of tubular absorption, it has become the mode to explain intracellular accumulation in the tubule as the result of absorption of materials from the lumen fluid. But the difficulties in the way of acceptance of this mechanism in the case of the normal "fatty infiltration" of the cat and dog kidney become even greater when the abnormal accumulations in the tubule cells of lipoid nephrosis in man are examined. In Figure 15 is shown a complete unstained nephron from a kidney of this condition. The brightly refractile fat distends the cells of the proximal convolution and the degree of this infiltration may be appreciated by its contrast with the non-fatty, almost invisible delicate contours of the ascending limb and distal convolution which twine about it. But in the proximal convolution there is no regularity of deposit, for though all its cells contain fat, in the middle of its terminal portion the deposition abruptly becomes so intense that the tubule is thrown into a mass of tortuously kinked, swollen coils (Fig. 15a) beyond which it again assumes its distended but regularly contoured course. To assume that such a localized distortion could be the result of suddenly increasing absorption from the tubule lumen would seem to raise more difficulties than it settles. One hesitates to speak of cellular "degeneration" in these days, but there does seem to be something "wrong" with the tubule cells.

Figure 16 shows the details of the appearances at higher magnification of infiltrated proximal convolutions when stained with cadmium-Sudan III mixture. In the upper convolution is seen an even dilatation of cells, so filled with fine fat droplets as to obscure all cellular detail. In the lower tubule the "bouffant" effect is not due, as would appear at first glance, to the development of diverticula, but to herniations of swollen cell substance through the confines of the limiting membrana propria. Here again it would appear certain that some intracellular alteration must be the origin of any such pressure from infiltrating fat. A simple exaggeration of a normal absorptive process seems at least inadequate.

DISTURBANCES IN THE HANDLING OF MINERAL SALTS

The example of a disturbance in the metabolism of a mineral salt most commonly seen in the kidney is the precipitation of Ca salts in its tissues. Whenever cells of the tubule die from any cause, they may, if local metabolic conditions are proper, become the seat of calcification. The example of the calcified debris in the sublimate kidney is a well known example.

In other instances a general disturbance in the metabolism of Ca and P seems the primary trouble, such as that associated with functional hyperactivity of the parathyroid. We have not as yet been able to examine the renal lesions of these conditions, but have noted the occurrence of deposits of calcium salts when phosphates are added in considerable amount to the diet of rats (16). Under these circumstances the cells of the terminal portion of the proximal convolution become heavily infiltrated with Ca salts. Most unfortunately, as will be evident in what follows, our observations were made by means of histological sections only. In a later study of the same problem Mac Farlane in Glasgow (17) confirming the deposition of the Ca in the renal cells, placed the site of deposit in the ascending limb. To settle this disagreement as to what part of the nephron is involved, a later dissection of the nephrons by us and staining of the cells and Ca in the continuity of the complete specimen definitely showed that both experts were both right and wrong. In Figure 17 it can be seen that we had missed the deposits in the middle portion of the ascending limb seen by Mac Farlane and that the latter had overlooked the accumulations in the proximal convolution observed by us (Fig. 18). It may be noted in passing as an example of the nicety of the metabolic localizations observed in the tubule of the nephron, that the deposits of Ca are never present in that terminal portion of the ascending limb where glycogen accumulations are found. Again, then, we see that method settles immediately what might have become, as it was in the case of glycogen deposition, a minor polemic extending interminably as one investigator after the other put on record the results of a technique inadequate to his problem. The application of the method to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calcification seen after the experimental administration of parathormone and viosterol may lead to similar clarifications in our present theory of the action of these substances in parathyroid disease.

DISTURBANCES IN THE HANDLING OF PROTEINS

The question as to the degree of permeability of the glomerular membrane to protein is quite naturally and properly answered by the physiologist and the pathologist each according to his peculiar convenience. To the physiologist it may be for practical purposes impermeable to protein and the glomerular filtrate can so be regarded as a protein-free ultrafiltrate. The pathologist, remembering that no other living membrane is protein-retaining, that mammalian glomerular fluid, when actually tested, can at best be said to contain not more than 0.025 per cent of protein

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FIG. 17. An ascending limb from the kidney of a rat fed on excess of phosphate. At the junction of the middle and lower third of the tubule is a swollen fusiform segment in which the cells are necrotic and infiltrated with calcareous material. The remainder of the tubule is normal except for the beginning of a similar lesion at the top of the figure. Magnification 150 ×.

FIG. 18. To the left a normal ascending limb from the kidney of the same rat. Note the normal pattern of the mitochondria. To the right is the terminal segment of the proximal convolution, the cells of which are infiltrated with calcareous salts. There is considerable necrosis and desquamation of these calcified cells in the upper portion of the tubule. Magnification 150 ×.

FIG. 19. Beginning and end of a complete nephron from a rat the reduced kidney of which was excreting an excess of gelatin. To the left is shown the glomerulus and the first half of the proximal convolution. Its tubule is distended to a tube of flattened epithelium by long masses of coagulated serum and gelatin. Note the short collapsed empty segment in the center of the tubule. To the right the ascending limb of the same nephron passes upward to the loop of the distal convolution which then joins another connecting tubule to form the collecting tubule. This entire segment is similarly filled with coagulated protein and transformed into a thin tube of flattened epithelium. At the extreme right is another empty ascending limb and distal convolution in which the normal cellular pattern can be seen. Note that the protein has backed down a short distance into the connecting tubule of this unit. Magnification 55 ×.

and that even the most moderate exertion, such as walking, is commonly followed by its appearance in the urine in relatively gross amount, is inclined to believe that under the conditions of every-day practical existence some protein is quite regularly escaping into the glomerular filtrate. He is reinforced in this opinion by the thought that if only one 1/1000 of its concentration in the plasma were to escape into the glomerular filtrate, the concentrating effect of water absorption by the tubule would show it in the ultimate urine as a proteinuria of 0.6 per cent, unless some of it was absorbed by the tubule cells. The pathologist therefore is inclined to accept both glomerular passage and tubular absorption of protein as what might be called "quasi-normal" mechanisms.

There is a great deal of experimental support for the conclusion that the amphibian tubule can absorb protein (18) and some that this is true of the mammalian nephron. The evidence in the latter case is derived in great part from observations that granules and hyaline droplets within the tubule cells are associated with proteinuria (19) so that the old "cloudy swelling" of parenchymatous degeneration has become an "infiltration" and analogous, insofar as the external source of the intracellular protein is concerned, to that long recognized disturbance of fat metabolism. It is in explanation of the histological picture of "nephrosis" that these absorptive phenomena are drawn most heavily upon by the pathologist. It is possible however by the method of dissection to bring more objective data into the argument.

If gelatin is given intraperitoneally to rats it is promptly absorbed and excreted in large amounts in the urine. The kidneys of these animals increase as much as 30 per cent in weight and in histological section, a "protoplasmic disturbance" is seen within certain tubule cells that can be interpreted reasonably as evidence of tubular absorption of the gelatin.

If complete nephrons are stained, considerably more information is obtained. Against the black background of the heavily stained mitochondria are seen clear empty globule-like areas which represent the spaces occupied by the foreign material

previous to the acid maceration (Fig. 20). The gelatin is found only in the cells of the proximal convolution, but its distribution is specific, differing from that of the vital dye, of fat and of glycogen. Moreover, differences of localization may be demonstrated between different preparations of gelatin. Following the injection of a calcium gelatinate the deposition begins in low concentration near the glomerulus (Fig. 20, a), increases in the middle portion of the convolution (Fig. 20, b) and becomes intense in its terminal part (Fig. 20, c). After giving a polymerized gelatin, the cells of the first part of the convolution contain only a moderate amount, those of the middle portion the greatest concentration and in the terminal portion only traces are present.

An even more striking demonstration of the absorptive process can be obtained if the polymerized gelatin is stained with a dye that is firmly bound to it before it is injected. The compound can then be seen in an unstained nephron as deeply colored blotches of dye in the tubule cells. The distribution of this "vital staining" is, however, entirely different from that of the usual vital stains which I have described before, but is identical to that of the vacuolar spaces observed with unstained polymerized gelatin (Fig. 21).

A similar accumulation in the proximal convolution of proteins of many sorts can be shown by the same procedure. Horse serum, or serum albumin, for example, resembles in its distribution polymerized gelatin, the greatest concentration of the intracellular material appearing in the middle third of the convolution. In this region of maximum deposit the histological picture of extreme cloudy swelling with hyaline droplet formation is perfectly reproduced (Fig. 22, b), while in the terminal portion only traces of protein are seen (Fig. 22, c). In these preparations the protein was stained by the usual histological technique after it had been deposited in the cells, for it is not dissolved out of the tissues by the maceration as is the more soluble gelatin. Serum albumin may also be stained previous to injection and is then seen in colored form with the same distribution as that of the native material.

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Another abnormality in the handling of proteins by the nephrons that exaggerations of moderate leakage through the glomerular membranes may produce is the result of increase in the viscosity of the tubule fluid that occurs with increasing proteinuria. The mechanics of flow of the most dilute of glomerular filtrates down the narrow tortuous tubule is a matter of some mystery, for calculations by means of Poiseuille's law of the pressure required to produce flow, end with theoretical magnitudes which cannot be readily comprehended (20).

The practical results of increasing the viscosity of the tubule fluid above a critical point may be demonstrated experimentally; one has only to overload the nephrons with a protein that passes

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FIG. 20. To the left, a tracing of a complete proximal convolution from the kidney of a rat which was excreting calcium gelatinate. The small squares show the origin of the microphotographs. In a, the dark normal mitochondrial pattern of the tubule is preserved as there is little or no absorption of gelatin. In b, mid-portion of the convolution the closely packed globular clear spaces occupied by absorbed gelatin, now removed by solution, are seen replacing the dark background of the mitochondrial pattern, and in c, terminal portion, the cells are completely filled with the material and so appear as light bands outlining the relatively dark lumen of the tubule. Magnification 450 ×.

FIG. 21. To the left a tracing of a complete proximal convolution from the kidney of a rat excreting large amounts of polymerized gelatin which had been stained before administration with an azo dye. Instead of the intracellular spaces of the preceding figure the insoluble dye-gelatin compound is seen within the cells. In a, is seen a moderate concentration of absorbed dye-gelatin in the first part of the convolution; in b, the heavy concentration in the mid-portion; and in c, the low concentration and absence of absorption in the terminal segment of the convolution. Magnification 200 ×.

FIG. 22. To the left a tracing of a complete proximal convolution from the kidney of a rat excreting large amounts of protein following injection of horse serum. The insoluble protein within the cells has been stained post-mortem with iron hematoxylin. In a, is seen the low concentration of absorbed protein granules in the first part of the convolution; in b, the greatest concentration in not only granular form but also as "hyaline droplets," and in c, an almost complete absence of absorbed granules. Magnification 200 ×.

readily through the glomerular membrane. This can be accomplished by reducing the number of nephrons to one-fourth their normal number by nephrectomy and administering gelatin in an amount that was previously easily handled by the kidney. The animals die in a few days of uremia in anuria. On examining the nephrons in dissected specimens, it is seen that most of them are distended and filled from glomerulus to far down into the collecting system with masses of coagulated protein (Fig. 19). These coagula are not pure gelatin, for hemorrhage in the glomerular tufts has added not only serum proteins but red blood cells to the gelatin-saturated tubular fluid. The entire nephron and beyond is thus occluded by a "cast" of its lumen. The same results are obtained if the experiment is repeated with other proteins, such as horse serum or bovine albumin.

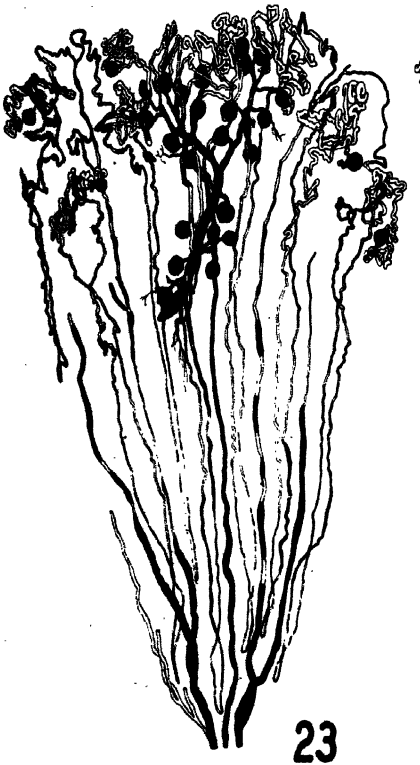
The phenomen of cast formation is one that has interested clinical students of Bright's disease ever since Henle first recognized the significance of their appearance in the urine. But the pathologists have contributed little to our knowledge of these objects and have for the most part rather discounted their importance in the pathogenesis of both functional and structural disturbances. Again we see the influence of a method which has directed the observers' attention away from structures that cannot be observed adequately by its technique, for it is not to be expected that much will be learned by random histological sections of objects that run through great lengths of long and tortuous tubule.

In the continuity of the dissected nephron the full significance of intratubular coagulation can be appreciated and the details of our first examination of the problem by these means will be found in another place (3). One result of this study is to be emphasized, for in it is found a clue to what follows, namely that primary casts or coagula are not found in all parts of the nephron indiscriminately, as the histological section would lead one to believe, but occur in the lower half of the nephron, in particular the distal convolution and collecting tubules. It is here evidently that conditions most favorable to intratubular coagulation of protein-containing fluid occur.

In recent years a considerable group of clinical disturbances of widely varied origin have been recognized which, beginning with no primary renal lesion, terminate in renal failure and anuria. When the kidneys of these cases are examined in histological section many of the tubules are seen to be filled with coagulated protein. Perhaps in part because of its lack of imaginative appeal, most clinical students have shied away from the obvious conclusion that fluid cannot flow readily through plugged conduits and searched elsewhere for their explanations of the oliguria or anuria. It is certainly true that an oliguria, due to lessened renal blood flow, might in certain cases well be the important antecedent factor in the causation of the coagulation, but the morphologist who depends on what he sees and what he can touch and feel perhaps more than on higher intellectual means of perception, can only believe that, in the last analysis, water cannot flow through stopped pipes.

Among the conditions where anuria and occlusion of tubules is observed are those where hemolysis *in vivo* has flooded the nephron with a tubule fluid rich in hemoglobin. This occurs after transfusion accidents and here the distal convolutions and the collecting tubules are found filled with brownish coagula. The appearance in the dissected nephrons and tubules is much more impressive than when viewed in the single slice of the histological section, for now the whole tortuous coil of a distal convolution is seen distended with coagulum or stretches of tubule in the collecting system a centimeter in length are completely occluded (Fig. 23). In blackwater fever the same appearances are noted, but in an exaggerated form. Practically all the distals and even ascending limbs are blocked as well as the greater part of the collecting system (Fig. 24). But even in this extreme example of intratubular coagulation the proximal convolution, though it may be filled with accumulations of debris from damaged epithelium does not show the presence of primary protein coagula.

Another condition in which occlusion of the tubule by coagulated protein has long been recognized is that accompanying the Bence-Jones proteinuria commonly associated with multiple my-



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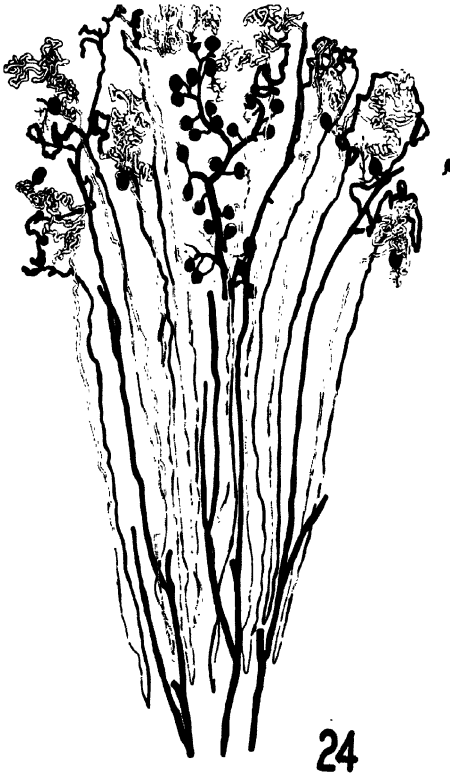

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elomata. Here great stretches of nephron tubule and of the collecting ducts are filled with solid occlusions of the protein body (Fig. 25) and the resulting distensions, deformities and atrophies are perhaps the most extreme alterations in renal architecture that are observed as a result of tubule blockage (Fig. 26). Here too is found an exception to the prevalent rule, for in certain cases, the proximal convolution is also distended and filled with the coagulated protein (Fig. 26, a, b).

There are two other conditions of interest in which occlusion of the nephron and renal functional disturbance have recently been noted. One is the kidney of the "crush" syndrome, where several days after the extensive crushing of tissue that not infrequently occurs in bombing, anuria develops and death occurs in renal failure. Dunn, Gillespie and Niven (21) have shown in sections of these kidneys that the ascending limbs and distal convolutions are filled with coagula of protein which contain blood pigments and that the tubule itself is also damaged, at times to the point of disruption.

In the dissected specimen these lesions stand out in striking clarity. In figure 27 portions of two distal convolutions are shown. In Figure 27, a, the intact evenly regular epithelium may

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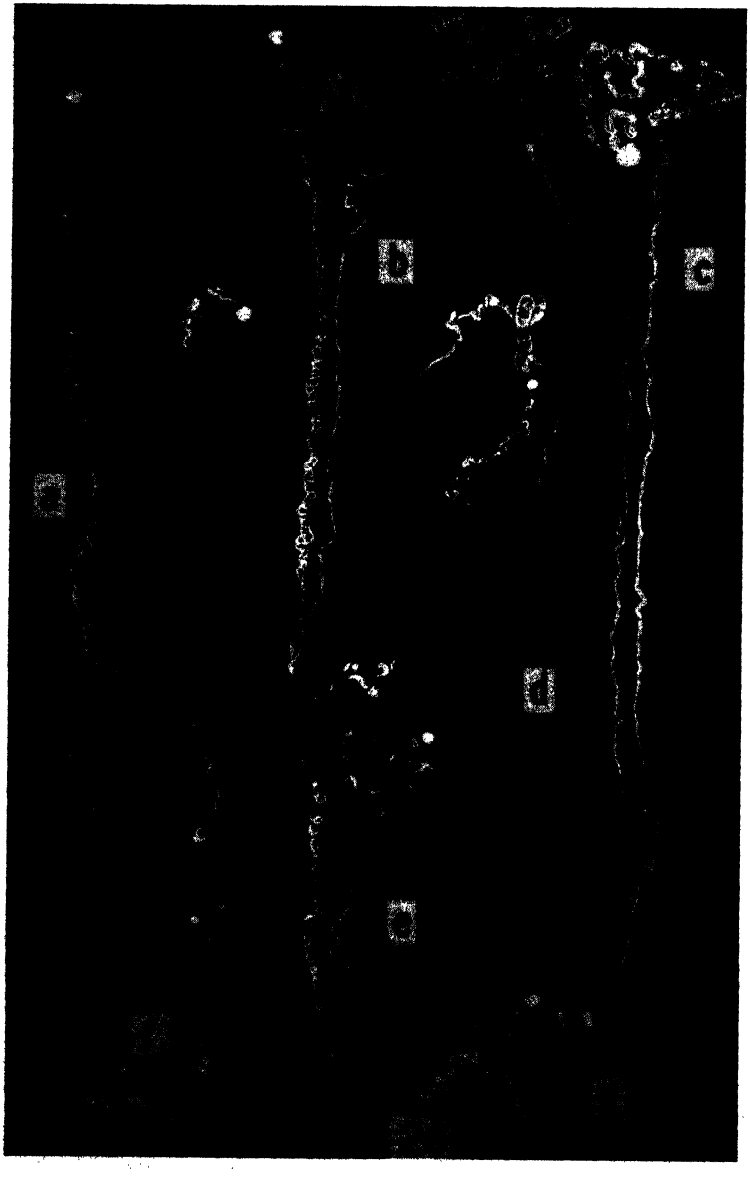
FIG. 23. Not a diagram but a collection of camera lucida tracings of actual nephrons dissected from the kidney of a transfusion accident. The arteries and glomeruli are shown in solid black as are occluding masses (casts) of hemoglobin-protein. Note that these occur only in the distal convolutions and collecting tubules. Magnification 6 ×.

FIG. 24. A similar preparation to the preceding figure from a kidney of blackwater fever with death in anuria. The black occluding masses in collecting tubules and distal convolutions are more extensive and run down into the ascending limbs of Henle's loop, but there are no coagulated masses in the proximal convolution. Note the dilatations of Bowman's space in the glomerular capsules. Magnification 6 ×.

FIG. 25. A similar preparation from a kidney of Bence-Jones proteinuria associated with multiple myelomata. The coagulations of this protein body are shown by cross-lined shading. Collecting tubules, and all parts of the nephron including the proximal convolution are filled with extensive occlusions. The details of the resulting distortions of the nephrons are shown in the next figure. Magnification 6 ×.



be followed from above downward to the point of rupture and here proliferating interstitial connective tissue surrounds and invades the disrupted tubule. Below this point there is less severe damage to the tubule wall and in the irregularity of its epithelium is seen the evidence of regenerative repair. In Figure 27, b, the lower half of the tubule is completely filled with a solid coagulum, whose deep tinge with hematoxylin shows its hemoglobin content.

A somewhat similar disruption of the distal convolution, though the intratubular coagulation is not so regularly present, has been described in histological sections following the accumulation of sulfathiazole crystals within the tubule (22). Here too the full significance of the lesion can be better appreciated by its appearance in dissected material. In Figure 28 a distal convolution, covered with diverticula, which bear no relation to the lesion, is shown in two magnifications. The crystals have disappeared in solution, but the disruption of the tubule wall is well shown by the fragments of epithelium scattered among the invading proliferation of connective tissue.

It is evident, therefore, that in many renal disturbances an important part of the pathogenesis of the structural lesion is an intratubular coagulation of fluid rich in protein; in Bright's disease this is a most important factor in the production of alterations in organ architecture (3), while in renal disturbances of the most varied origin it plays at least a subsidiary role in the production of functional disturbances. And similar coagulative phenomena must be responsible for the "casts" in the urine that

FIG. 26. Drawings of dissected nephrons from the kidney of the preceding figure showing the deformities produced by the occluding masses of Bence-Jones protein. In a, extensive distension with a solid mass of protein in the proximal convolution; in b, the terminal portion of the proximal convolution is occluded and there is a marked atrophy of its first part; in c, the coagulated protein distends the distal convolution while the proximal convolution is free and dilated; in d, the distal convolution is greatly distended and occluded by solid protein coagula and there is extreme atrophy of the nephron above this point. In e, the occluding mass fills the ascending limb. In f and g, distorted remnants of atrophic nephrons are shown. Magnification 12×.

are, along with the proteinuria, the classical evidences of renal damage. What, then, do we know of why and how fluid coagulates within the tubule lumen?

In 1895 the Danish biochemist K. A. H. Moerner (23) described the presence in normal urine of a substance which precipitates protein. An elaborately complete chemical study showed that this substance was not a nucleoprotein, that on hydrolysis a reducing substance was liberated from it and that analysis of the compounds which it formed with the precipitated protein contained N, C and S in approximately the proportions that would be expected if the precipitating substance was chondroitin sulfuric acid. Since chondroitin sulfuric acid was known to be a strong precipitant of protein Moerner concluded that it was the precipitating substance in the normal urine. Following Moerner's work there has been at times casual clinical interest in what was accepted as "chondroitinuria," but in none of these investigations was the chemical identity of the urinary substance examined until 1927 when Addis (24) suggested on the basis of chemical analysis of hyaline casts that they might be urinary protein precipitated by chondroitin sulfuric acid. Other biochemists, however, whose special interest has been the study of the muco-

FIG. 27. Distal convolutions from a kidney of "crush anuria." In the upper part of a the normal even pattern of the epithelium is seen while in its mid-portion the rupture of the tubule is evident with proliferation of the surrounding interstitial tissue which was so adherent to tubule at this point that it could not be removed by dissection. Below the irregularity of the regenerating epithelium is seen. In b, the tubule has not ruptured, though there are slight connective tissue adhesions about it. The upper half is lined with irregular regenerating epithelium, while in the lower half, the epithelium is compressed and obscured by a coagulum of hemoglobin containing protein. Magnification 180 ×.

FIG. 28. A distal convolution from a case of sulfathiazole damage. In its mid-portion the tubule has burst apart and fragments of its epithelial wall are seen lying among the proliferating connective tissue which invades the ruptured tubule. These details are better shown in the high power insert. Note the casual finding of diverticula on this distal convolution, in particular the huge pedunculated object which is filled with inspissated material. Magnification 54, 90 ×.

polysaccharides and glycoproteins, have been unable to identify chondroitin sulfuric acid in the urine. Doctor Karl Meyer tells me that in the concentrate of several hundred liters he was unable to demonstrate its presence.

In 1935 Lison (25) in his study of metachromatic staining showed in an extensive examination of both natural substances and substitution products especially prepared for his crucial experiments, that the property of inducing this reaction in toluidin blue is peculiar to those compounds of large molecular size that are in combination with the sulfuric acid radicle. Of tissue components the mucopolysaccharides, chondroitin and mucoitin sulfuric acid are the common examples of this nature. A confirmation of the essential details of these conclusions is found in the work of Bank and deJong (26). The dependability and practical usefulness of the metachromatic reaction may be appreciated when it is recalled that it was by its use that Jorpes was led to the identification of heparin as a mucoitin-sulfuric acid (27).

On the foundation of Moerner's and Lison's work certain simple experiments may be devised in which the presence of a precipitating body in the urine is tested, and the varying conditions are examined which occur in the tubule fluid and which might affect intratubular coagulation. The relation of these coagulative phenomena to the reaction of metachromasia can also be examined. The same procedures may then be used to compare the behavior of a weak solution of chondroitin sulfuric acid in regard to both coagulation and metachromasia. It will be understood that no rigorous physical-chemical examination of the complexities in behavior of protein solutions is to be expected from a morphological pathologist, and what follows is offered only as preliminary spade work which may disclose a field worthy of more competent labor.

In Table 1 the results of adding a solution of low concentration of protein (horse serum or serum albumin) to weakly acidified normal urine and to decreasing concentrations of aqueous solutions of chondroitin sulfuric acid are shown. It is seen that a faint precipitate forms in the urine, while a heavy precipitate is

observed with the chondroitin sulfuric acid which decreases to a degree comparable with that seen in the urine at a dilution of 1/40,000.

If the protein precipitates formed in these tubes are suspended in finely divided form in water and added to tubes containing a dilute solution of toluidin blue the metachromatic reaction becomes visible. In tube 5 of Table 1 the protein precipitate was heat-coagulated serum and so serves as a control for the series, for it will be observed that the dye retains its orthochromatic pure blue tone. In the second, third and fourth tubes with the protein precipitate by chondroitin sulfuric acid is seen the full development of the reddish-violet tone of metachromasia. In the first tube the unwashed protein precipitate from the urine shows a distinct alteration in tone but this is far from the frank reddish-violet of a full metachromatic reaction. If this urine precipitate is washed several times in distilled water and then tested in the dye solution, full metachromasia is observed. Evidently then there are urinary substances in the precipitate, readily removed by washing, that interfere with the reaction. It will be remembered that the precipitation of protein in the presence of normal urine was also very slight, a point to which we shall later return.

TABLE 1

Protein Precipitation by Normal Urine and Chondroitin Sulfuric Acid

1cc. urine (Chond. SO_4) + 1 cc. 1/64 serum + 1 cc. 1/10 acetic

		Ppt.	Metachrom.
Tube 1.	10 cc. normal urine	+	+*
Tube 2.	10 cc. 1/10,000 Chond. SO_4	++	++
Tube 3.	10 cc. 1/20,000 "	++	++
Tube 4.	10 cc. 1/40,000 "	+	+
Tube 5.	10 cc. H_2O—(boiled)	+++	–

* Metachromasia indefinite until after washing precipitate.

Explanation of metachromatic symbols in all tables.

++ = strong violet-red.
+ = definite reddish-violet.
± = faint violet tone of blue.
– = pure blue (orthochromasia).

An examination of factors present in normal urine that interfere with the development of metachromasia is shown in Table 2. The first tube serves as a control showing the true blue of the orthochromatic dye. To all the other tubes of toluidin blue a relatively large concentration, 1/10,000, of chondroitin sulfuric acid has been added, along with various urinary constituents. The second tube does not contain any urinary substance, but is made in aqueous solution and in it fully developed violet-reddish metachromasia is seen. The remaining tubes all show a modification of the pure orthochromatic blue, but in none has the chondroitin sulfuric acid produced its full effect. The tube containing urine

TABLE 2

Interference with Metachromatic Reaction

				Metachrom.
Tube 1.	10 cc. H_2O	+ Toluidin blue	+ No Chond. SO_4	–
Tube 2.	10 cc. H_2O	"	1/10,000 Chond. SO_4	++
Tube 3.	10 cc. urine	"	"	green
Tube 4.	10 cc. urine salts	"	"	±
Tube 5.	10 cc. 1.6% urea	"	"	±
Tube 6.	10 cc. dial. urine	"	"	±

has a greenish cast from its yellow pigment; the "urine salts," sodium phosphate and sulfate in aqueous concentration approximating that of normal urine of 1.020 specific gravity, and the tube with 1.6 per cent aqueous urea are a muddy off-tone of blue, while urine dialyzed to a practically chloride-free content also prevents any more than a moderate change in the color of the dye. Evidently then, both dialyzable and non-dialyzable substances in the urine inhibit the metachromatic reaction with chondroitin sulfuric acid. It would be interesting to know, therefore, if the dialyzable substances also interfere with the development of the coagulative phenomena when precipitation of protein is occurring in normal urine.

The dispersive action of urea comes to mind. A sample of normal urine containing 1.6 per cent. urea was divided into two

parts and the urea removed from one by the action of urease. When the two samples were tested by adding serum and dilute acetic acid, only a slight cloudiness formed in the untreated urine, while a definite precipitate formed in the sample from which the urea had been removed.

An even greater change becomes apparent, however, when the urinary salts as well are removed from the urine. In Table 3 is shown the increasing precipitate of protein from acidified normal urine that has been dialyzed against running water for increasing periods of time until its specific gravity is reduced as indicated. The fourth tube, dialyzed for 24 hours, had a specific gravity of 1.000 and was negative to $AgNO_3$ for chlorides. With the decrease in specific gravity there goes increasing precipitation of protein. Testing the washed precipitates shows a strong metachromatic reaction. Returning to the first tube of the series, which contained undialyzed urine of a specific gravity of 1.020 it is found that although precipitation had occurred to only a slight degree, there had nevertheless been more reaction between some urinary constituent and the added protein than meets the eye, for shaking with $CHCl_3$ produces a considerable flocculent precipitate. If tested with toluidin blue it is found metachromatic. As controls to the "denaturing" effect of the shaking with $CHCl_3$ no precipitate is produced in normal "protein-free" urine and only traces of precipitate are obtained with an aqueous solution of horse serum. If these traces are tested with toluidin blue they are not metachromatic. It is evident therefore, that, as the metachromatic reaction is "inhibited" by presence of dialyzable elements in the urine, so also are the coagulative phenomena. This interference is apparently more concerned with the stability of the altered protein than with the reaction of the protein with the precipitating factor. Moreover, it is seen that the element, or elements, responsible for protein coagulation are non-dialyzable. For the sake of economy of words I shall refer to this substance, or substances, as X-body in what follows.

The non-dialyzable property of the X-body, and parenthetically it will be remembered that chondroitin sulfuric acid is non-

TABLE 3

Interference with Protein Coagulation by Dialyzable Urinary Constituents

50 cc. urine + ½ cc. serum + 1 cc. 1/10 acetic

		Ppt.
Tube 1.	50 cc. urine sp. gr. 1.020	±*
Tube 2.	50 cc. urine dial. to 1.010	+*
Tube 3.	50 cc. urine dial. to 1.005	++*
Tube 4.	50 cc. urine dial. to 1.000	+++*
Tube 5.	50 cc. H_2O	–

Tubes 1 and 5 shaken with $CHCl_3$

1 = ppt. ++* 5 = ppt. ±**

* Metachromatic. ** Orthochromatic.

dialyzable, allows us to test its resistance to heat, and finding it heat-resistant, to remove salts and urea and some pigments and concentrate it in dialyzed urine. In tubes 1, 2, 3 and 4 of Table 4 are seen the precipitating effects of samples of dialyzed normal urine the increasing concentration of which was produced by evaporation at a temperature just below boiling. In other experiments where evaporation occurred in cellophane tubes before a fan at room temperature somewhat stronger percipitating reactions were observed than occurred with heat concentrated urine, but for the practical purposes of our experiments the X-body may be considered heat resistant.

TABLE 4

Concentration of Non-dialyzable Urinary Precipitating Factor

50 cc. urine + 1 cc. 1/10 acetic + ½ cc. serum

		Ppt.
Tube 1.	50 cc. dial. urine 1 ×	±
Tube 2.	50 cc. dial. urine, heat conc. 1.5 ×	+
Tube 3.	50 cc. dial. urine, heat conc. 2.0 ×	++
Tube 4.	50 cc. dial. urine, heat conc. 4.0 ×	+++
	Urine mixtures of sp. gr. 1.010	
Tube 5.	50 cc. aa-4 × dial. urine + orig. urine	++
Tube 6.	50 cc. orig. urine dial. to 1.010	+

With increased concentrations of the X-body available, it is now possible to show that the degree of inhibition of coagulation by the dialyzable materials is a matter of relative concentration between the inhibiting substances and X-body. For example, the same concentration of dialyzable inhibitors present in the same urine of a specific gravity of 1.010 may be prepared in two ways, either by diluting the original urine of a specific gravity of 1.020 with an equal volume of four times concentrated dialyzed urine whose specific gravity is 1.000 or by dialyzing the same original urine to a specific gravity of 1.010. In the former case there is two and a half times the concentration of the precipitating X sub-

TABLE 5

Effect of Protein Concentration on Precipitation by Urine Factor

4 cc. dialy. 4 × urine + 1 cc. serum dil. + 0.2 cc. acetic

1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
0	0	0	0	++	++	++	+	0
				+	+			

1 cc. 0.03% Chond. SO_4 + 1 cc. serum dil. + 0.2 cc. acetic

1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
0	+	++	++	++	+	0	0
		+	+				

stance as in the latter while the concentration of dialyzable substances, as measured by the specific gravity, is the same. As seen in tubes 5 and 6 of Table 4 under these conditions a much heavier precipitate of protein occurs in the former. The importance of this demonstration will perhaps be more evident when we consider the application of our theories to physiological actualities, for it shows that even in a urine of moderately high specific gravity, 1.010, coagulation can occur, if the concentration of the X-body is raised.

It is possible also to demonstrate that relative concentrations of other substances in the coagulating system affect the precipitating reaction. In Table 5 the addition of an excess of protein is shown. It is seen that there is an optimum relative concentration between the protein and the urinary precipitating factor and

that with an excessive amount of protein no coagulation whatever occurs. A similar relation is shown in the precipitating reaction with chondroitin sulfuric acid.

As would be expected the coagulation of protein by the urinary X-factor is influenced by the concentration of H ions. In Table 6 is seen the precipitating effect of a constant unit of the urinary factor acting on a constant concentration of various proteins but at a varying pH obtained by use of phosphate-citric acid buffers.

TABLE 6

Effect of H Ion Concentration and Isolectric Point of Protein in Precipitation by Urinary Factor

1 cc. protein sol. + 1 cc. dial. 4 × urine + 1 cc. buffer

	pH								
	3.4	4.2	4.8	5.2	5.6	6.4	7.0	7.4	7.8
Serum	++*	+	±	0	0	0	0	0	+†
Frac. V .	+++	+	0	0	0	0	0	0	+
Frac. IV	+++	+	+	+	0	0	0	0	+
Frac. II	+++	++	++	+	+	+	0	0	+
B. J.	++	+	+	+	+	±	±	±	+

* Metachromasia. † Orthochromasia.

The proteins used were horse serum and certain of the plasma protein fractions kindly supplied to me by Dr. Cohn. These preparations allow an examination of the behavior of many of the protein bodies which are found in proteinuria. Fraction V is largely serum albumin, IV–3.4 is about one-half β globulin and one-half α globulin, and fraction II, 95 per cent γ globulin. The isoelectric points of these fractions are, serum albumin 4.7, II, circa 7.2, and that of IV–3.4 in the neighborhood of 5.5. A sample of urine containing Bence-Jones body was also used as a protein solution, since there are reasons, judging from the localization of the intratubular coagula, for supposing that it is precipitated in the kidney tubules under somewhat special conditions. Its isoelectric point is usually given as 5.8.

It will be observed in Table 6 that the precipitation of the protein by the urinary X-body lies in each case on the acid side of the isoelectric point and that since this point varies with the different protein bodies a range of precipitation from 3.4 to 7.0 occurs. This behavior is the expected; what is less readily understandable is the precipitate that occurs with all proteins at a pH of 7.8. That some action of the urine is involved is shown by a control series, similar to those of the table except that the urine is replaced by water, for at no pH is any precipitate formed with any of the protein bodies. The reaction is therefore not due to the H ions or the salts of the buffer. The occurrence of this anomalous precipitate is however of some use to us even if we do not understand it, for if the metachromatic reaction of all the

TABLE 7

Effect of Protein Concentration on Degree of Metachromasia

50 cc. dial. urine + 1 cc. 1/10 acetic + serum

	1 cc. serum	½ cc. serum	¼ cc. serum
Metachromasia	+	++	+++

precipitates is tested, it is found that those forming on the acid side of the isoelectric point are all positive and thus give evidence of a combination of some sort between the protein and the X-body, while that forming on the alkaline side is entirely negative, indicating that the precipitate was free of X-body.

And finally to complete the demonstration of the analogies and similarities of the two reactions, coagulation and metachromasia, it is seen in Table 7 that the concentration of protein affects the degree of metachromasia that develops in the urine- protein precipitate in a manner analogous to its action on coagulation. To a constant amount of dialyzed urine increasing amounts of horse serum were added. Precipitation occurred in all, most copious when the lesser amount of protein was added. Equal amounts of these protein precipitates were washed and suspended in water and the degree of redness, i.e., of metachromatic change, compared in a colorimeter. It is evident that metachromasia though

present in all, increases with decrease in the amount of protein in the precipitate. There is therefore an analogy with the action of excess protein in the precipitating reaction.

The findings of these *in vitro* experiments may be summarized as follows. Protein-containing urine is a system in which stability is determined by equilibria involving: a, the concentration of dialyzable substances (electrolytes, urea); b, the concentration, and nature, of the protein; c, the concentration of H ions; and d, the concentration of a non-dialyzable heat-resisting body (X-body). Increasing concentrations of the dialyzable substances and of the protein tend to maintain stability, increasing concentrations of H ions and of the non-dialyzable body (X-body) tend to coagulation.

The behavior of the system to the metachromatic reaction is analogous to its behavior in regard to dispersion and coagulation. Orthochromasia is the analogue of dispersion and metachromasia of coagulation.

The coagula of protein and X-body are strongly metachromatic; coagulations of protein by heat and salts are not.

A similar behavior both in the coagulative and metachromatic properties is shown if chondroitin sulfuric acid, which Moerner claims to have demonstrated in normal urine, is added to a solution of protein.

An excursion into what of personal necessity was an oversimplified examination of the physical and chemical mechanisms of intratubular coagulation has led us far from the morphology of the nephron. But pathologists are often forced into such hazardous digressions and since the justification they give for these journeys into foreign fields is that it allows them to return with some comprehensible tale to their domestic morphological domain, they must be forgiven their wanderings. Providing of course that they do return, and in the present instance the bridge that makes possible this much-desired passage from the complexities of function to the simplicities of structure is the metachromatic reaction. For we have seen that not only does this reaction afford presumptive evidence of the presence of an essential and

until now unappreciated component of the intratubular coagulating system, but also that the mechanisms of the testing reaction itself are influenced by the same factors as favor or inhibit the coagulating processes which are our primary problem.

The tissues, either in histological section or in dissected material, can be stained with toluidin blue and, under proper conditions, the metachromatic properties of its elements determined. To those who wish to use the method a careful examination of Lison's studies (25) is recommended, for the reaction, as was evident in the *in vitro* tests, is influenced by many factors which must be controlled if significant results are to be obtained.

In Figure 29 a frozen section from a kidney of chronic glomerular nephritis stained with toluidin blue shows dilated tubules filled with hyaline coagula. The coagula vary considerably in color, as did the precipitates from protein-containing urine, though in most the reddish tone of metachromasia is evident. To what intensity the reaction may develop is seen in Figure 30 where collecting tubules deep in the medulla are shown in cross-section. As a control is seen the orthochromatic blue-tinged content of the tubule to the left of center, which is filled not with a protein coagulum but with an accumulation of cellular debris.

The tissue elements also show in varying degree the reddish tones of the positive reaction. We shall limit our observations at this time to a consideration of those tissues of the kidney immediately concerned with the coagulated material, that is the wall of the tubule, and such observations can be best made in dissected material.

In Figure 31 stained with toluidin blue, are shown the beginning and end of a nephron from a rat's kidney that had been excreting large amounts of protein following the intraperitoneal injection of horse serum. The cells of the proximal convolution are filled with absorbed protein, similar to the previous demonstrations that you have seen, and it will be observed that this protein gives the true blue tone of orthochromasia. Only in the terminal tip of the convolution is the violet hue of meta-

chromasia evident in the protoplasm of the tubule cells, while in the ascending limb and the distal convolution the reaction in the tubule wall becomes pronounced. In Figure 32, a, the junction of distal convolution, connecting and collecting tubule is shown at higher magnification, and here although the protoplasm of all the epithelium is definitely violet, the heavy granules of the intercalated cells are more strongly positive.

In this kidney many nephrons were occluded in their distal convolutions by large cast-like coagula. Figure 32, b, shows one of these with the compressed epithelium of the tubule wall and the metachromatic reaction of the occluding coagulum.

Although many questions still remain unanswered, such as, for example, the source of the metachromatic substance and how it becomes concentrated in the cells of the distal nephron, the morphological evidence so far presented allows, I believe, a tentative description of why and how intratubular coagulation occurs.

The reason as to why coagulation occurs can be found in the addition to the formerly incompletely described intratubular system of protein, water, salts and H ions of the equally essential metachromatic X-body. Coagulation is not to be considered due to it or to any one of these various factors but is to be regarded as the result of equilibria that determine the stability and hence the coagulation of the tubule fluid.

How these equilibria are established within the nephron can be found in the degree of protein leakage through the glomerulus, the extent of its absorption by the proximal convolution, the absorption of water and of salts, reciprocally reflected in alterna-

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FIG. 29. Kodachrome microphotograph of frozen section stained with toluidin blue of kidney in chronic glomerular nephritis. The dilated tubules are filled with coagula (casts) which show by their varying reddish-violet tones various degrees of the metachromatic reaction. Magnification 150 ×.

FIG. 30. A similarly treated frozen section from the medulla of the same kidney showing the intensity of reddish tone in the strongly metachromatic casts in the collecting tubules. The large tubule is filled with heterogenous debris, not a true coagulative cast, and shows the blue tones of orthochromasia. Magnification 150 ×.

Fig. 29

Fig. 30

Fig. 31

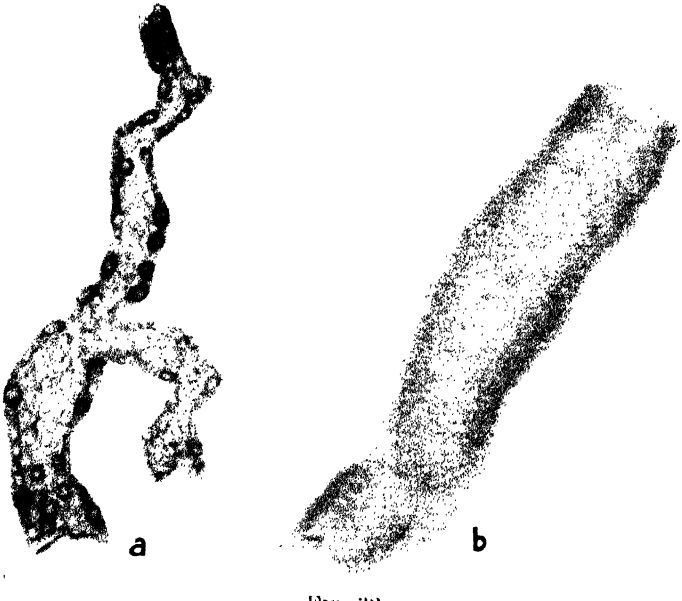

Fig. 32

tions in the specific gravity of the fluid, the increasing acidity that reaches its maximum in the distal convolution and finally the increased concentration of the X-body in both lumen and epithelial tissues of the distal nephron. The occurrence of these known physiological variables shows how the equilibria may shift with resulting stability or coagulation even while our knowledge of the exact details of both *in vitro* and *in vivo* conditions are still incomplete.

As a practical example, we can understand the usual localization of casts and coagula in the distal nephron and collecting system. This cannot be due to water absorption, the greater part of which has occurred in the proximal convolution where coagulation rarely occurs (11, 3), or acidity, for both are demonstrably inadequate to cause coagulation of the proteins, which in the common proteinuria are a mixture in which serum albumin predominates over the globulins. The high isoelectric point necessary for this coagulation is not attained until the fluid reaches the distal nephron and at that point, the concentration of X-substances being high, coagulation occurs. In the less common proteinurias where globulins with low isoelectric points predominate, such as the Bence-Jones proteinurias, coagulation may occur higher in the nephron with a lower acidity and at a lesser concentration of X-body.

FIG. 31. Dissected proximal and distal convolutions stained with toluidin blue from the kidney of a rat that was excreting large amounts of protein after administration of bovine albumin. In the cells of the proximal convolution is seen much absorbed protein in the form of granules which present the true blue of the orthochromatic reaction. In its terminal tip a faint violet tinge of metachromasia becomes evident. In the distal convolution there are no granules of absorbed protein and renal cells show the strong violet-red tone of metachromasia. Magnification 75 ×.

FIG. 32. From the kidney of the same animal. To the left two connecting tubules join to form a peripheral collecting tubule. All of the epithelial cells show violet metachromasia, but the intercalated cells stand out prominently by the more intense reaction of their granules. Cf. Figure 7. To the right a distended distal convolution is filled with a hyaline coagula of precipitated protein. This cast and the compressed epithelium beneath it show the violet tone of metachromasia. Magnification 200 ×.

Speculation of the sort in which I am indulging may perhaps be permitted if it is remembered that the title of my lecture made no promise of arriving at factual conclusions, but indicated only a desire to show the directions that extension of our knowledge in renal morphology may take by use of methods where continuity of structure is maintained. I trust that there has been enough in this lecture of accomplished fact, some of it complete so far as it goes and more as yet only roughly sketched in provisional form, to make one definite conclusion. The conclusion is that the structural aspect of renal activity offers as rich and limitless a field to the investigator as does the functional. All that is needed are methods appropriate to the task.

The value of that conclusion is of course not limited to its application in renal problems. The human mind, being what it is, can think of a structure without function, dead, if you will, but existent, but it cannot conceive of free and disembodied function without structure, except in the realm of metaphysic. We can have something doing nothing, but not nothing doing something.

Granting then the present-day intensity of the expanding drive of functional dynamics in the field of pathology, must there not be corresponding improvement in the structural machine that is to bear this ever-increasing functional load? High octane in the model T will get us nowhere, unless it is in the ditch. And to whom are we to look for advance in structural concepts if not to the pathologist? It would seem then that if he is to become a biochemist, then the biochemist must become a morphologist and such a circle I am sure, as a pathologist, could be peculiarly vicious. At times tonight you may have glimpsed a prevision of its dangers.

In any case, those who still delight in the wondrous and varied aspect of Form in its adaption to function will not despair. For Form has still its ancient implication, *forma,* beauty, and as a wise and most eloquent morphologist has said "whatsoever is most beautiful and regular is also found to be most useful and excellent" (28). If the application of the

oldest of scientific techniques, the one first known to primitive man, the picking of things apart, can still afford results to which your Society can so patiently listen, what has the morphologist to fear for the future, with his newfound electron microscopes and microspectrographs? For him, too, the new world has its promise.

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CHEMICAL AND BIOLOGICAL RELATIONSHIPS BETWEEN HEXOSES AND INOSITOLS[1]

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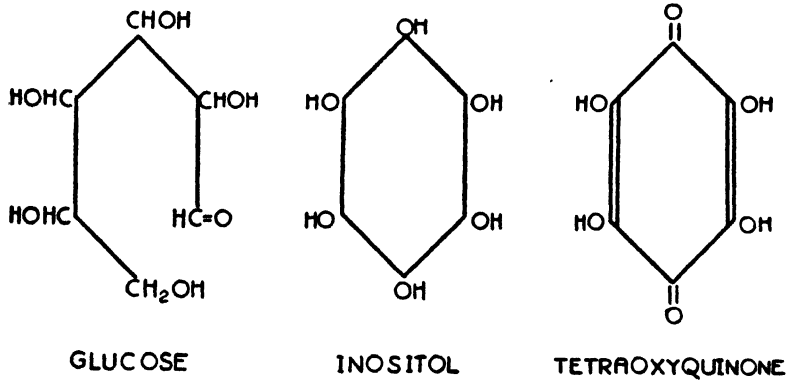
SINCE its discovery in 1850 by Scherrer, inositol has been considered by chemists to be an intermediate between carbohydrates and aromatic substances. The reasons are that on the one hand it has the same empirical formula ($C_6H_{12}O_6$) and the same sweet taste, and solubility in water as sugar; in fact, Scherrer called it muscle sugar. On the other hand it is oxidized by nitric acid to tetrahydroxy-quinone demonstrating the easy transition to the aromatics.

TABLE 1



As it was not possbile at that time to verify by chemical means the transition from sugar to inositol, this class of compounds disappeared for quite some time from the limelight of chemical research.

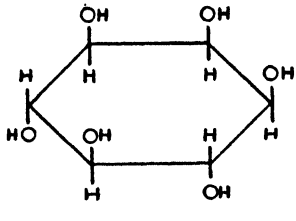
A general interest in inositol was revived through discoveries in the biological field. In 1928, in Toronto, Miss E. V. Eastcott

[1] Lecture delivered January 18th, 1945.

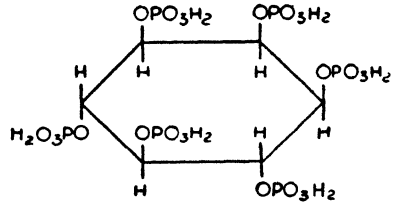
(1) demonstrated that meso-inositol is required for the growth of certain yeasts. Her experiment has since been amply substantiated and is now used as the basis of Dr. D. W. Woolley's quantitative method (2) for estimating inositol. In 1942, R. J. Williams and his co-workers (3), using almost the same method, amplified the data on the distribution of inositol. Its very frequent appearance in animal and plant tissues seems to presuppose important physiological functions. Recently, G. W. Beadle (4) published another bioassay method for inositol using an "inositolless" strain of Neurospora crassa. It is apparent, however, that this method is practical only for rather high concentrations of inositol.

A number of isomers and analogs of inositol are present in nature. These isomers do not exhibit any biological or nutritional action, and therefore I shall confine myself mainly to the discussion of the biologically active member of the family, the *meso* inositol. It occurs in nature in at least four forms: free inositol, phytin, lipositol (5) and a water-soluble non-dialyzable complex (6).

TABLE 2



MESO-INOSITOL



PHYTIC ACID

Free inositol has been isolated from many plant and animal sources. Phytin, the calcium and magnesium salt of inositol-hexaphosphate, was believed until recently to be exclusively a constituent of plants. Its presence in many seeds has been known for decades.

The steep liquor of the corn refining process is at present the best industrial source in America for inositol and phytin. In 1940, S. Rapoport (7) showed that the erythrocytes of species

like the chicken and the turtle, in which these cells are nucleated, contain appreciable amounts of phytin. Since 1930, inositol as a constituent of special phosphatides has been known from the work of R. J. Anderson (8), who isolated it from the phosphatides of the tubercle bacillus. In 1939, Klenk and Sakai (9) obtained inositol monophosphate from soya bean phosphatides. The widespread occurrence of inositol in phosphatides was also evidenced in 1942, when J. Folch and D. W. Woolley (10) demonstrated that it is a constituent in the cephaline fraction of brain and spinal cord and thus recognized a new inositol-containing phosphatide, in animal tissues. In 1943, D. W. Woolley (5) isolated a similar compound from soya beans and demonstrated that it was composed of inositol monophosphate in glucosidic linkage with galactose and combined with ethanol amine, tartaric acid, oleic acid and saturated fatty acids. Woolley named this compound *lipositol.*

The occurrence of inositol as an integral part of a phosphatide and the role of inositol in the prevention of fatty livers brings to mind, as Dr. Woolley points out, the occurrence of choline in a phosphatide, and its vitamin-like action in the prevention of fatty livers of a different character. Perhaps the formation of *lipositol* is one of the uses to which dietary inositol is put by the animal. In 1941, Woolley (2) recognized a water-soluble nondialyzable inositol complex in liver and in 1942 he showed that inositol complexes were present in most of the tissues that were examined.

These are a few of the more important examples illustrating the significance of this compound in the animal body. More detailed information may be found in an excellent review paper by Dr. D. W. Woolley (11). This article also contains a comprehensive description of the nutritional significance of inositol. It is quite evident, from this paper and from other sources, that there is no unanimity of opinion as to whether or not inositol should be regarded as a dietary essential. For instance, in his studies of purified diets for mice, Woolley (6) showed that a severe alopecia developed, which could be cured or prevented by

inositol. However, the picture was confusing, since alopecia also developed when they were fed rations low in pantothenic acid. Moreover, spontaneous cures of inositol deficiency occurred frequently (12).

Following Woolley's paper on alopecia, Pavcek and Baum (13) reported that inositol cured "spectacled eyes" in rats reared on a purified diet. It is a common observation, however, that in many laboratories rats reared on purified diets, apparently free of inositol, do *not* develop any loss of hair around the eyes.

Martin (14) has presented evidence which indicates that an inositol deficiency may be precipitated by including p-aminobenzoic acid in the diet. Animals receiving thiamin, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, choline, inositol and p-aminobenzoic acid as supplements to purified diet, developed normally. The removal of either inositol or p-aminobenzoic acid resulted in poor growth and changes in the pelt. When *both* were removed the animals again grew normally. However, Barnett Sure (15) reported experiments practically duplicating those of Martin, except that the levels of vitamins were different, where he found that neither inositol nor p-aminobenzoic acid had any effect on the growth of young rats.

A clear-cut case of inositol deficiency in rats is described by Cunha, Kirkwood, Phillips and Bohstedt (16). A severe alopecia developed in rats reared on a natural diet composed of corn and soya bean meal, which could be cured by inositol.

The role of inositol in the nutrition of the lactating rat is similarly in dispute.

Climenko and McChesney (17) found that on purified diets either with or without p-aminobenzoic acid, the inclusion of inositol increased the milk yield of lactating rats. The inclusion of p-aminobenzoic acid alone seemed to have an *adverse* effect on lactation. The reverse of this finding has been reported by Barnett Sure. Supplementing the diet with p-aminobenzoic acid greatly improved lactation while inositol was decidedly injurious.

These differences in findings cannot yet be reconciled.

A further interesting feature of inositol is its lipotropic action,

which was reported by Gavin and McHenry (18) in 1941 in Toronto. These workers produced fatty livers in rats by feeding a beef liver fraction. Choline did not affect the fatty disposition but both lipocaic and inositol were effective in preventing its development.

Interest in these findings has been revived by the studies of Abels, Kupel, Pack and Rhoads (19), who showed that inositol apparently has a lipotropic effect on human beings also. It is well known that patients with gastro-intestinal cancer usually have fatty livers. Inositol defats such livers. In this connection the work of Laszlo and Leuchtenberger (20) might also be mentioned. These investigators reported that the daily intravenous injection of minute amounts of inositol prevented the development of transplanted tumors in mice.

Thus there have been many contradictory findings about the action of inositol. When a chemist studies these findings his reaction is more or less as follows:

Here is a supposedly pure substance which presents such a confusing picture in its nutritional application that he suspects it to be *not quite pure.* Polyalcohols with numerous OH groups, in the case of inositol six, have a decided tendency to adsorb both organic and inorganic foreign material which cannot be removed by ordinary crystallization. It is possible to produce derivatives of inositol with completely blocked OH groups which can be distilled in a high vacuum. After distillation they can be reconverted into free inositol by hydrolysis. Inositol purified in this way has been prepared in my laboratory and is just now being tried out by the Banting-Best Department of Medical Research of the University of Toronto, as to its effect on fatty livers, in comparison with the commercially available inositol. These experiments have not as yet been completed. If the action of the two samples of inositol differs, it would indicate that the commercially available inositol made from the steep water of the Corn Products Refining process contains some highly biologically potent factor X adsorbed by the inositol. D. W. Woolley (6), in 1941, made similar experiments in the study of alopecia. He pre-

pared meso-inositol hexacetate, purified it by frequent recrystallization—but not by distillation—and hydrolyzed the hexacetate to inositol. Inositol, thus treated, did not differ from commercially available inositol in the treatment of alopecia.

These instances, in which inositol seems to be important for biological procedures, are well known.

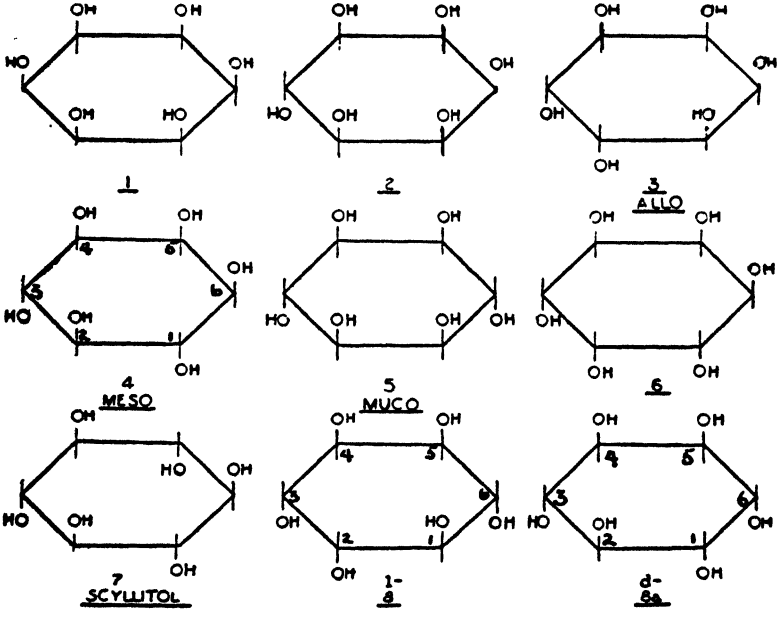
I would like to draw your attention to a further occurrence of inositol, described by A. Portmann (21), of Basel, Switzerland. Portmann points out that fish, especially the shark, have comparatively large amounts of inositol in the muscles of their fins, but no glycogen or other reserve carbohydrates in the liver. He believes that inositol is probably a reserve carbohydrate formed from glucose and stored in the fins, thus providing a quickly available source of glucose for the blood stream, just by the reopening of the inositol ring.

Another case where inositol may play the role of a reserve carbohydrate, has been presented by L. B. Winter (22), in a paper on "The Inositol Content of the Mammalian Heart." It might be assumed that the relatively high amount of inositol found by this author to be present in the heart muscle of the ox suggests the possibility that it functions as a reserve carbohydrate, providing energy for this muscle.

There are other instances where animals have been shown to synthesize inositol. As early as 1858, Vohl (23) was able to isolate quantities of inositol from the urine of a man with diabetes insipidus far in excess of the amount which we now know could have been obtained from the food. The extra inositol excreted probably arose from synthesis. Following this lead, Needham (24) in 1924 showed that rats rendered polyuric by administration of salt excreted more inositol than was ingested. Since the excretion of this excess of inositol continued over long periods the conclusion was that the rats were synthesizing the substance. Woolley (2) demonstrated that mice fed a synthetic diet frequently synthesized inositol in amounts almost equal to the minimal effective dose of the compound for this species. At least one point of origin of this synthesized inositol was shown to be the

intestinal flora of the mice. The great problem for the chemist is, of course, to determine from which material the inositol is synthesized. He naturally believes that it is from *glucose,* and I shall now present some chemical evidence to bolster this view. My co-worker, Mrs. Gerda Dangschat, and I were prompted to make an investigation of the problem through the means of organic chemistry, the more so because we had successfully cleared up similar relations between certain plant acids such as

TABLE 3

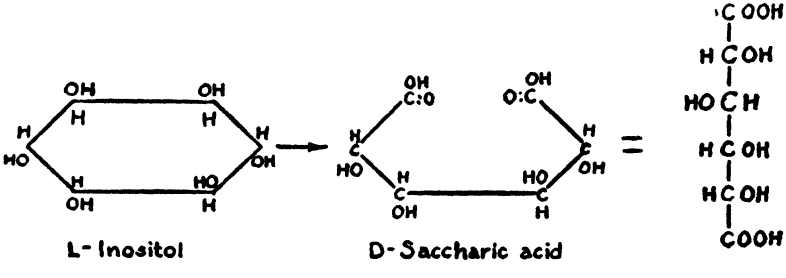


shikimic acid (25) or quinic acid (26) and glucose. In the case of these acids we were able to trace clearly the connection with glucose by determining the configuration of their asymmetric carbon atoms. On perusal of the literature on inositol we learned to our surprise that as late as 1939 the configuration of this long-known substance had not been determined. Admittedly, there had been in existence since as early as 1894 a very elegant treatise

by Bouveault (27) on the theoretically possible inositols, but no correct experimental data establishing the configuration of the most important member of this group, namely, the meso-inositol.

From Table 3 it can be seen that there are seven configurational possibilities for an inactive inositol, Nos. 1–7. The possibilities 8 and 8A must be assigned to the optically active members of this group, namely d and l inositol, first prepared from natural sources by Maquenne (28). In 1936 Théodore Posternak (29), in Geneva, Switzerland, proved the formula 8 for l-inositol by oxidation of the compound to D-saccharic acid (Table 4). If num-

TABLE 4



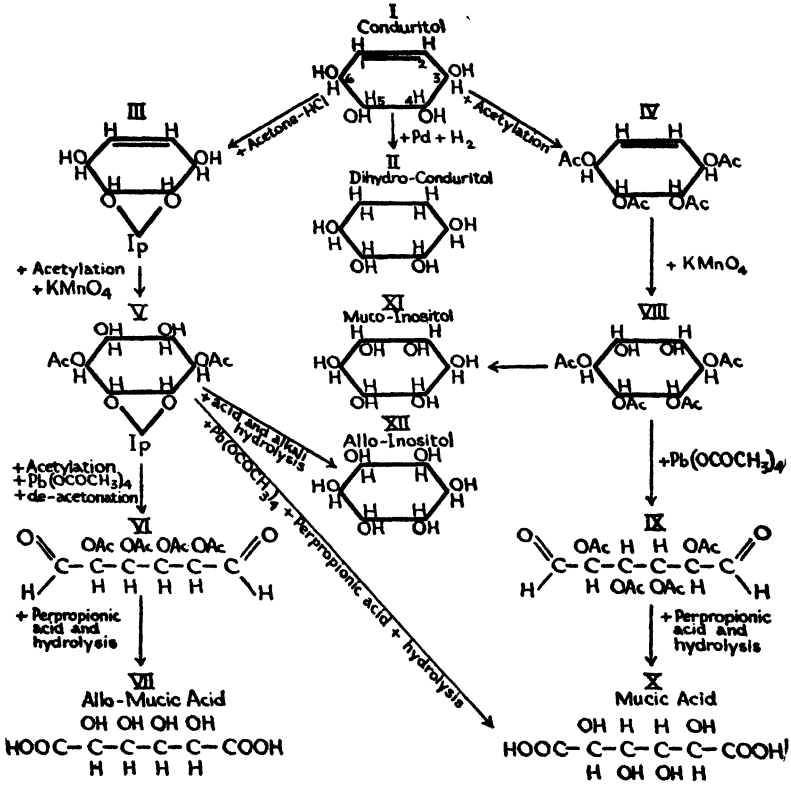
ber 8 is l-inositol, this leaves formula 8A for d-inositol. The configuration of the two optically active inositols (8 and 8A) was thus firmly established by Posternak's work. However, the seven inactive inositols were still undetermined. Obviously, the application of the usual methods of sugar chemistry for the determination of their configuration should be made. One creates a point of attack by singling out one specific pair of hydroxyls by combining the polyalcohol with formaldehyde, acetone, benzaldehyde and the like. None of the usual methods worked, so we began working in a circuitous way using a material offered by nature which already contained a suitable point of attack, namely, a double bond. The material used was a compound called conduritol (30) isolated from Condurango bark and already known to be a tetrahydroxy cyclohexane.

Table 5 is comprised of the series of reactions by which we were enabled firstly to prove the configuration of conduritol, and sec-

ondly, to transform conduritol into the two well-defined synthetic inositols, muco-inositol (XI) and allo-inositol (XII).

We arrived at the constitution of conduritol by acetonating it with acetone and hydrochloric acid to a monoacetone conduritol III. Compound III being indifferent against lead tetra-acetate

TABLE 5



must carry the iso-propylidene residue in the OH groups of the carbon atoms 4 and 5. After acetylation, III is oxidized by means of potassium permanganate and soda to V.

Following acetylation in 1 and 2, and removal of acetone from 4 and 5, the ring of V is opened with lead tetra-acetate, between

carbon atoms 4 and 5, yielding the dialdehyde VI. This dialdehyde is easily transformed by means of perpropionic acid and hydrolysis into *allo-mucic* acid VII. The configuration of carbon atoms 1, 2, 3 and 6, in compound V is thus proved.

The transformation of conduritol I into compound VIII and thence into mucic acid X is achieved in a similar manner, the sole difference being that in place of a diacetyl *monoacetone* conduritol derivative a tetra-acetate of conduritol IV is oxidized by means of potassium permanganate and soda. This operation yields compound VIII in which the newly formed cis hydroxyl pair is located on the opposite side of the ring plane in contrast with compound V. This latter fact is proved by the opening of the ring of VIII with lead tetra-acetate resulting in the dialdehyde IX.

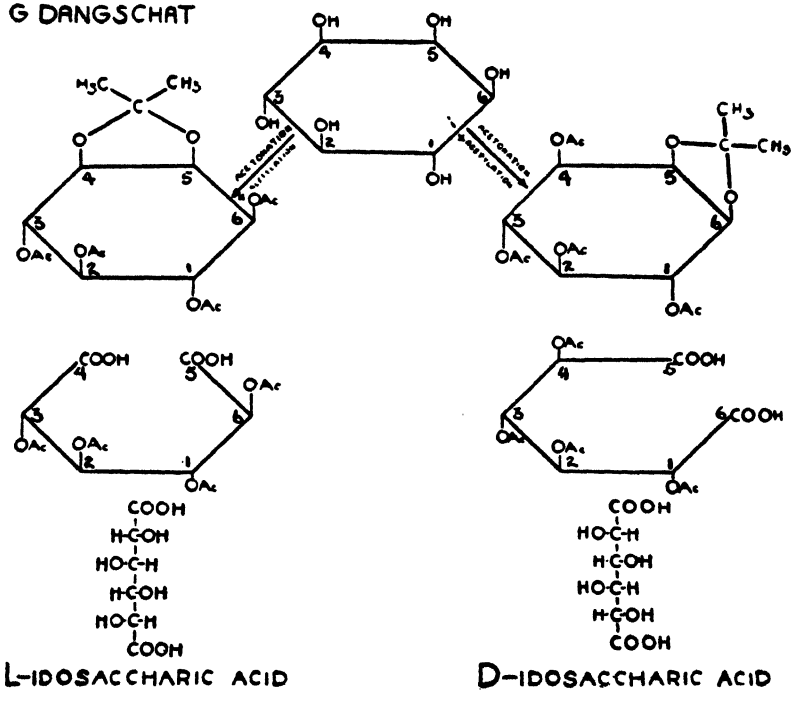
Compound IX is transformed into *mucic acid* X by oxidation with perpropionic acid and subsequent hydrolysis. The formation of mucic acid obviously proves the configuration of carbon atoms 3, 4, 5 and 6. This result, together with the proof of configuration by the formation of allo-mucic acid, completes the cycle. Therefore, the configuration of conduritol and the compounds V and VIII is proved. V and VIII can easily be hydrolyzed to their underlying free inositols, namely, muco inositol XI and allo inositol XII. These are so called because of their configurational connection with mucic acid and allo-mucic acid.

This presents an interesting example of the arbitrary choice for attachment of a hydroxyl pair to the double bond of a cyclohexane derivative either in cis or in trans position. We may assume that the explanation for the differing behavior of compound IV and the diacetylated compound III towards potassium permanganate lies in the fact that the ring of compound III is distorted to some extent by the secondary iso-propylidene ring, whereas compound IV is undistorted.

At this point we were rather unhappy, because neither of the two well-defined inositols which had been prepared with great effort was by any means identical with meso-inositol, the configuration of which we wanted to determine. They are merely

artificial products useful only for disposing of 2 of the 7 theoretical possibilities in the series of inactive inositols, and consequently on the whole, a disappointment. In order to determine the configuration of meso-inositol, Mrs. Dangschat and I attempted another procedure for the acetonation of meso-inositol.

TABLE 6



This time we were successful (31). We were aware from the work of Meerwein (32) and from our own experience that zinc chloride with one molecule of an organic acid, e.g., acetic acid, forms a so-called ansolvo acid, an extremely powerful acid catalyst for acetonations. Inositol, boiled with a large excess of acetone containing some zinc chloride and acetic acid yielded a monoacetone meso-inositol which in the form of its tetra-acetate

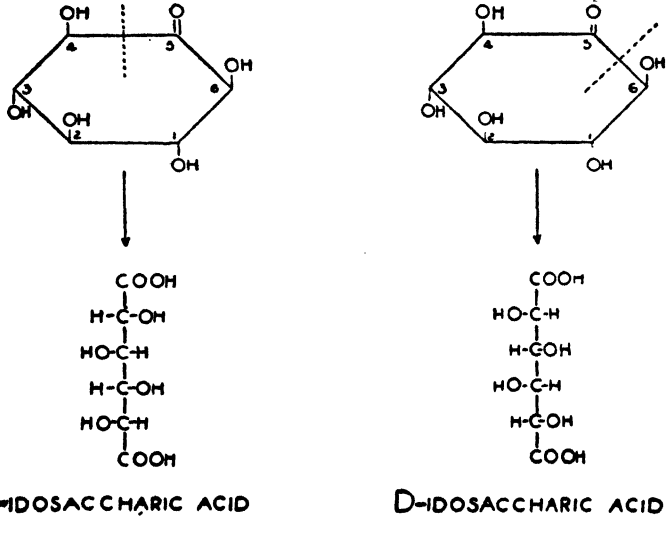
could be distilled and isolated. The acetone was removed and the ring opened by means of lead tetra-acetate.

In greater detail: the monoacetone inositol is a mixture of two enantiomorphic forms. The molecule of the meso-inositol contains three OH groups on the same side of the ring, and consequently, it is possible to block pair 4 and 5 or the pair 5 and 6 by

TABLE 7

M. POSTERNACK

BIO-INOSOSE ACCORDING TO KLÜYVER AND BOEZAARDT [1939]

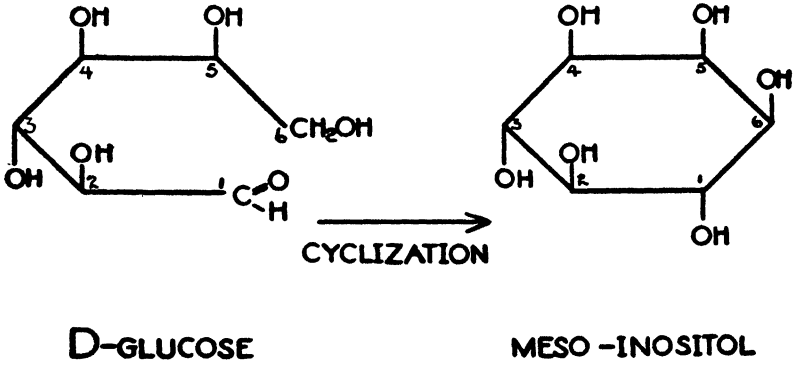


acetone. When, after acetylation, the acetone is removed by mild acid hydrolysis, we have again two enantiomorphs, one with two free hydroxyls in 4 and 5, and one with two free hydroxyls in 5 and 6. Treatment of this racemic mixture with lead tetra-acetate, therefore, opens the ring between 4 and 5, and between 5 and 6. One can see from Table 6 that equal amounts of D-ido-saccharic acid and L-ido-saccharic acid, are formed. The intermediate step, namely, the formation of the corresponding dialde-

hydes, being self-evident, is omitted. The dialdehydes are oxidized to the corresponding acids as previously, by treatment with perpropionic acid.

For the formula of meso-inositol, the following conclusions can be drawn: The two hydroxyls which can be blocked by acetonation are vicinal and on the same side of the ring. If, D,L-ido-saccharic acid is formed when the ring is opened between the two hydroxyls 4 and 5 or 5 and 6, then the other four hydroxyls must be alternatingly on the one side or the other side of the ring plane,

TABLE 8



and one of them vicinal to the hydroxyl pair which can be blocked by acetone. Thus the formula of meso-inositol was proved convincingly for the first time. Three months later Théodore Posternak (33), then in Geneva, now in Lausanne, Switzerland, confirmed our results in the following manner: As a starting material he took inosose, a keto-inositol, prepared by Kluyver and Boezaardt (34) from inositol through the action of acetobacter suboxydans in 1939 (Table 7). Inosose yielded about 20% D,L-ido-saccharic acid on oxidation with soda alkalic potassium permanganate, which means that the ring of the inosose is split left or right of the keto group 5, forming D-ido-saccharic acid, as indicated on the right side of Table 7, and L-ido-saccharic acid, as indicated on the left side of Table 7. Posternak's results show

also that acetobacter suboxydans oxidizes meso-inositol specifically on the carbon atom 5, which carries the middle group of the three vicinal OH groups on the one side of the ring.

At this point I should like to say something about the numbering of the carbon atoms of inositol used here. This is by no means arbitrary but is influenced by the idea that at least in several instances inositol is formed from glucose, so we have numbered the six carbon atoms of inositol analogously to the usual numbering of the carbon atoms of glucose. Obviously, the pair of hydroxyls in cis position which can be acetonated is borne by the carbon atoms corresponding to numbers 4 and 5 in glucose. Hereby, the designation of the other four is given, and we must assume that a cyclization similar to an aldol condensation occurs between 1 and 6, as shown in Table 8.

If two new asymmetric carbon atoms are formed by means of an aldol condensation, they tend to have the opposite configuration, which amounts in the case of the inositol ring to a trans position of the hydroxyls concerned. The hydroxyls on the carbon atoms 1 and 6 are actually in trans position. This is a further reason in support of the idea that inositol may be formed by the ring closure of glucose.

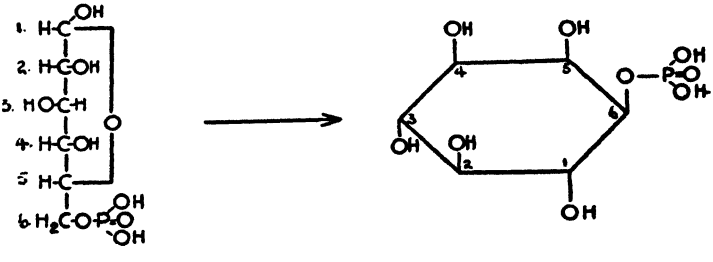
A pertinent example of the creation of two asymmetric carbon atoms of opposite configuration by means of an aldol condensation is described by H. O. L. Fischer and E. Baer (35). We found that upon condensation between dihydroxy-acetone and D-glyceraldehyde, the ketoses D-fructose and D-sorbose were formed in a yield of almost 50% for each, whereas the theoretically possible D-psicose and D-tagatose could not be detected. This illustrates our point that the newly formed asymmetric carbon atoms in position 3 and 4 have opposite configuration. We chose to call this phenomenon "the non-occurrence of isomers," and are of the opinion that it may play a considerable role in future inositol chemistry.

The aforementioned manner of numbering reveals at once the very interesting fact that acetobacter suboxydans oxidizes in meso-inositol that hydroxyl group on carbon atom 5 exactly as

it oxidizes in glucose the OH group on carbon atom 5 in the well-known preparation of 5-keto-gluconic acid from glucose or gluconic acid. One is tempted to say that not only the chemist but also the "bug" considers inositol to be a glucose in disguise.

The possession of a keto-inositol with the keto group in position 5 enabled Posternak (33) to make a further experiment. He reduced the ketose with sodium amalgame in acetic acid and achieved two isomers which differed only in the configuration on carbon atom 5. The one proved to be identical with meso-inositol, the configuration of which is already known, and the other was identical with *scyllitol.* Scyllitol differs from meso-inositol only

TABLE 9



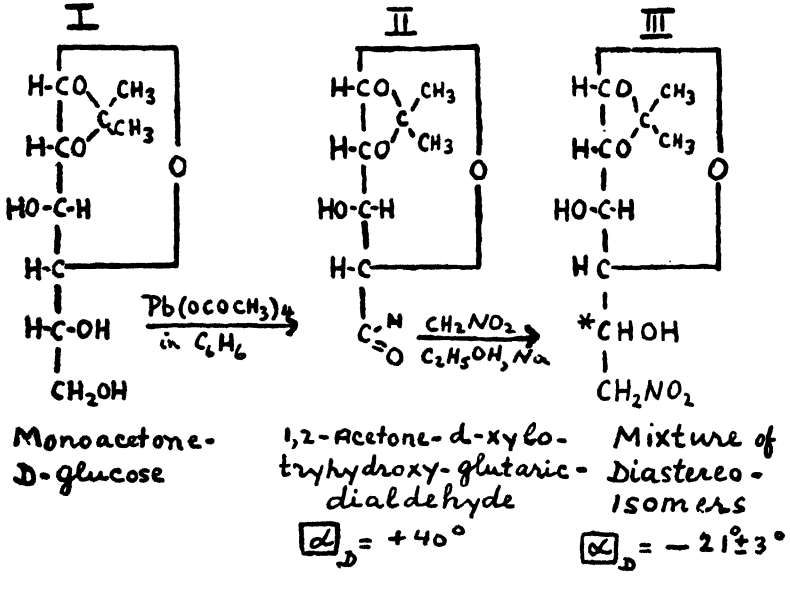
with regard to the configuration in 5, so it is represented by formula 7 in Table 2. Thus four of the seven possible formulae for an inactive inositol are proved.

So, finally in 1942, the configuration of meso-inositol was established independently in two laboratories. This substantiates our idea that meso-inositol may be formed by cyclization of glucose. Of course, I do not propose that this cyclization takes place in nature with free inositol, but incline to the belief that the CH_2OH group in 6, is mobilized in nature by phosphorylation and the cyclization takes place under the influence of a suitable enzyme. This idea, as represented by Table 9 is entirely hypothetical.

As soon as circumstances permit, I intend to work in a laboratory on the Pacific coast in an attempt to purify the suspected

enzyme from the fins of shark and to endeavor to transform glucose-6-phosphate, Robison ester, into a phosphate of inositol. This enzymatic experiment has been necessarily postponed. However, an experiment in which glucose could be cyclized to inositol *by purely chemical means* was suggested and successfully carried out by Dr. J. M. Grosheintz (36) in our laboratory in Toronto. In his series of reactions the activation of carbon atom 6, which in

TABLE 10

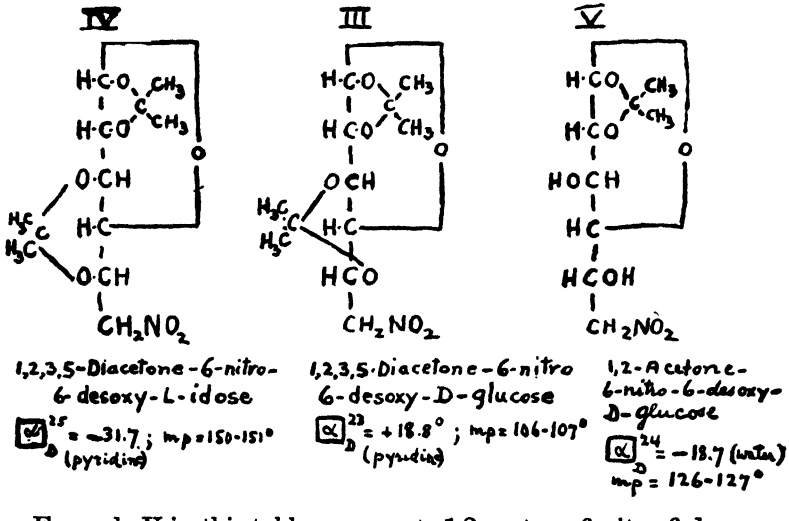


nature is supposedly afforded by phosphorylation, was effected by the introduction of a carbon bound nitro group. It was demonstrated that 6-nitro-6-desoxy-D-glucose and 6-nitro-6-desoxy-L-idose cyclize almost quantitatively to a mixture of nitro-inositols. For the preparation of these nitro-hexoses the following reactions were used (see Table 10):

According to Koichi Iwadare (37), monoacetone D-glucose was treated with lead tetra-acetate splitting out formaldehyde and producing the compound II which is actually a sugar dialdehyde

of the five carbon series with one open and one blocked aldehyde group. When this dialdehyde was treated with nitromethane and alkali a mixture of the two diastereo-isomers, theoretically to be expected, resulted. These two differed from each other only in the configuration on carbon atom 5, which is indicated in Table 10 by a star. Now Dr. Grosheintz was confronted by the rather tricky task of separating these two very similar isomers and he finally succeeded in achieving it by a method which we like to call "fractional acetonation." The formulae pertinent to this operation are shown in Table 11.

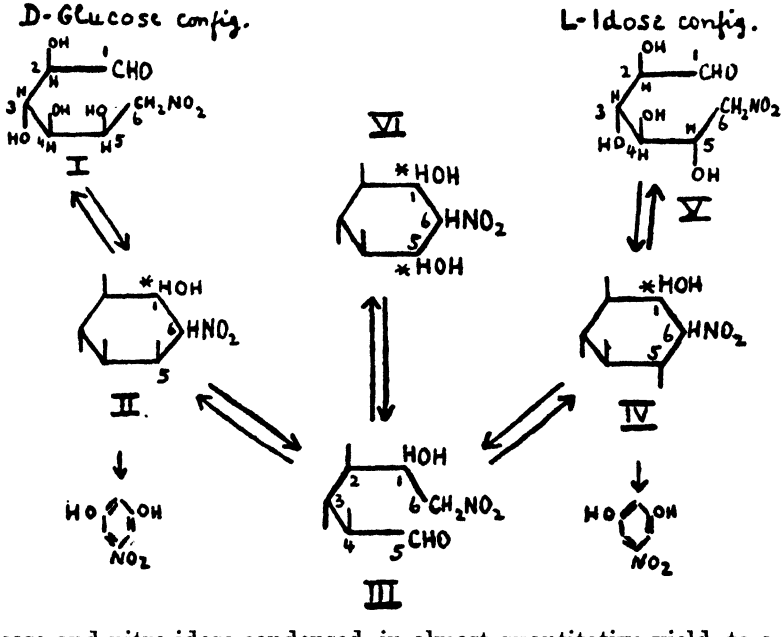
TABLE 11



Formula V in this table represents 1,2-acetone-6-nitro-6-desoxy-D-glucose. It was apparent that the acetonation of the free hydroxyl groups in compound V, and the formation of its diacetone derivative, represented by formula III could be effected only with difficulty. On the other hand, the corresponding idose had the hydroxyls on carbon atoms in 3 and 5, in "cis" position, and these could be easily acetonated forming the compound IV—1,2,3,5 diacetone 6-nitro-6-desoxy-L-idose. We were able—although some loss was incurred—to remove the idose from the

mixture of idose and glucose derivatives, by treatment with acetone and hydrochloric acid. The glucose derivative remained to a large extent undissolved. This was, of course, not an ideal separation, but it served our purpose. By removal of the acetone groups, free 6-nitro-6-desoxy-D-glucose and -L-idose were prepared. The decisive cyclization experiment is represented by Table 12. In slightly alkaline solution, both the free nitro-glu-

TABLE 12



cose and nitro-idose condensed, in almost quantitative yield, to a mixture of *only 2 nitro-inositols.* In every instance these same two nitro-inositols resulted, no matter whether glucose or idose was used as a starting material. As indicated in Table 12, an equilibrium seemed to exist between the possible forms of nitro-inositols. In this equilibrium the ring could be closed and opened between carbon atoms 1 and 6, just as effectively as between carbon atoms 5 and 6. The difficult and laborious separation of

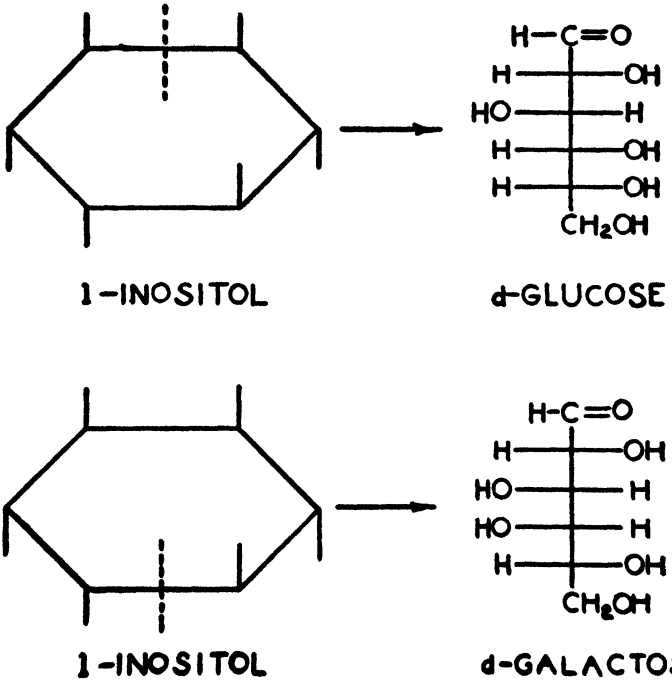
the glucose and idose series, as described above, was of no avail for the purpose of cyclization. Therefore, in ensuing experiments we worked with the mixture, 6-nitro-6-desoxy-D-glucose and 6-nitro-6-desoxy-L-idose.

The configurations of the carbon atoms 2, 3 and 4, in the nitro-inositols were proved to be the same as those of the corresponding atoms of glucose. It will be necessary to determine those of carbon atoms 1, 5 and 6 by the familiar methods of sugar chemistry at a later date. This, however, does not in any way affect the salient point, which is *that for the first time the ring closure of a glucose derivative to an inositol derivative had been accomplished.* We contend that such a model experiment as ours gives validity to the belief that inositol may be formed in nature through cyclization of the always readily available glucose.

Furthermore, it might be noted that nitro-inositols presented a good example of easy transformation from the cyclo-hexane to the aromatic series. Under very mild conditions, e.g., on treatment with pyridine and acetanhydride, our nitro-inositols aromatize, yielding, almost quantitatively, diacetyl 5-nitro-resorcinol (38). A similarly smooth transition had been observed by Posternak, who described the action of the same reagent on inosose, as yielding tetra-acetyl-1,2,3,5 tetrahydroxy benzene (39). It is known also that hydroaromatic acids, such as quinic acid, react in a similar way (40). The biological material on the transformation of glucose into inositol—presented earlier in this lecture—together with the purely chemical evidence of the same reaction, lends support to the idea that the six carbon sugars will easily form an inositol ring, and that again, the inositol ring, under suitable conditions will open, re-forming hexoses. An enlargement of this idea arouses some rather challenging speculations. The inositol rings opened by biological means, e.g., by the action of enzymes on inositol phosphates, could form most of the theoretically possible hexoses, if the opening occurs between appropriate carbon atoms. For biological considerations we are solely interested in the naturally occurring hexoses. Let us imagine, for instance, that D-glucose and D-galactose could be formed

from l-inositol (Table 13), and similarly, D-mannose and D-galactose from d-inositol (Table 14). Through the D-galactose, d- and l-inositols would be interconvertible. The logical conclusion which could be drawn is that all three naturally occurring hexoses could be interconverted through the medium of the

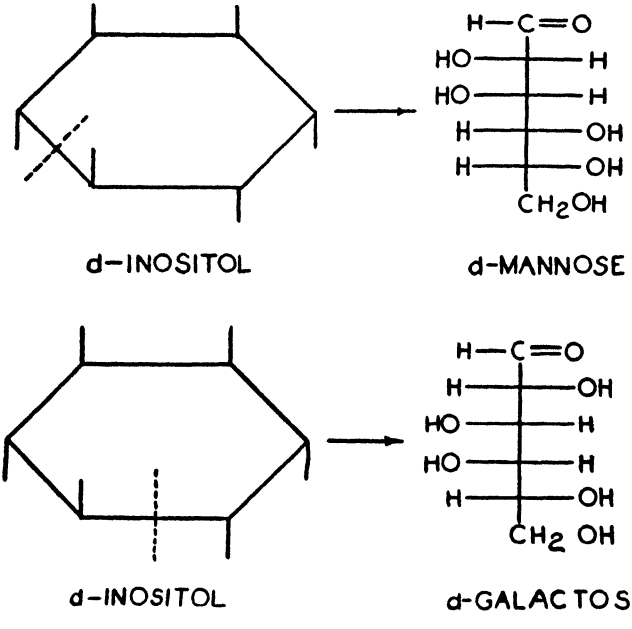
TABLE 13



inositols. All of this is merely "paper chemistry," which, however, might be close enough to reality to constitute a useful working hypothesis. We should note, however, that the configuration of the most widely occurring member of the inositol family, namely, meso-inositol, does not suggest any interconversion of hexoses. The only hexose occurring in nature which will form meso-inositol, by cyclization, and which can be formed from meso-inositol by ring splitting, is D-glucose.

To summarize: Aside from the biological possibilities for vitamin-like action, it could be deduced from the chemical evidence presented, that inositol is most likely an intermediate between carbohydrates and aromatic substances; moreover, it very likely serves as a reserve carbohydrate, storing away glucose in a form which could be easily mobilized. Finally, there is the speculative

TABLE 14



possibility that inositol could act as an intermediate enabling the easy transformation of one hexose into another.

It is possible that we are just beginning to recognize the usefulness and versatility of inositol in the household of nature. The purpose of this lecture has been to show that everything organic chemists are able to contribute—namely, model experiments and determination of the configuration of the products concerned—may be useful tools in aiding the solution of problems of biochemistry associated with inositol.

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THE GENETIC CONTROL OF BIOCHEMICAL REACTIONS[1]

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THE evidence that genes are intimately concerned with the control of specific chemical reactions of the organism has increased rapidly in the last decade. As an example of the nature of this evidence, the hereditary disease in man known as alcaptonuria is pertinent. Alcaptonurics differ from normal persons in being unable to degrade the compound 2,5-dihydroxyphenyl acetic acid (homogentisic acid or alcapton). Instead of undergoing its normal breakdown, probably through acetoacetic acid to carbon dioxide and water, this substance is excreted in the urine by alcaptonurics. It gives rise to a dark pigment on oxidation and it is this property that is responsible for the characteristic symptom of alcaptonuria, darkening of the urine on exposure to air. This symptom was described three hundred years ago and the chemical basis of it was discovered eighty years ago when homogentisic acid was isolated from the urine of alcaptonurics and identified chemically. At the turn of the century it was suggested that this particular metabolic defect is inherited as a simple Mendelian recessive trait. A dozen years later it was shown that alcaptonurics lack a specific enzyme concerned with the breakdown of homogentisic acid and present in the blood of normal individuals. It appears that alcaptonuria results initially because a particular hereditary factor or gene is defective or absent. As a result a specific enzyme is likewise defective or absent. Under these conditions the particular chemical reaction by which homogentisic acid is broken down does not occur. These and other facts and interpretations concerning alcaptonuria are summarized by Garrod in his book "Inborn Errors of Metabolism" (1), a classic of both biochemistry and genetics that in the past has not been appreciated fully in either field.

[1] Lecture delivered February 15, 1945.

This type of relation between gene and chemical reaction, known for thirty years in the case of alcaptonuria, is apparently a general one. Many alterations in metabolism which result from single gene substitutions can be similarly interpreted in terms of defects in specific chemical reactions. In man, to consider further examples, there is known a recessive Mendelian trait in which phenylpyruvic acid is excreted in the urine instead of being oxidized to its parahydroxy analogue, as it apparently is in normal individuals (2, 3). A most significant consequence of inability of the individual to carry out this particular reaction is a severe mental defect—phenylketonurics are invariably idiots or imbeciles. This example illustrates the important point that advances in our understanding of the basic metabolism of the organism can be made through the use of genetic defects. If a deliberate attempt had been made to correlate phenylalanine metabolism with the functioning of the nervous system in normal individuals, it is most improbable indeed that the importance of the phenylpyruvic acid → *p*-hydroxyphenylpyruvic acid transformation would have been appreciated. It is of course a long way from this specific reaction to a complete undersanding of the chemistry of rational thinking, but it is at least a first step. The part the defective gene has played in the taking of this step is obvious and we would be quite blind to the possibilities for future progress if we did not recognize that further steps are possible in the same way.

Medes (4) has described one individual who was physiologically unable to carry out the oxidation of *p*-hydroxyphenylpyruvic acid to its 2,5-dihydroxy analogue. Since only a single individual of this type has been recorded, we have no means of telling whether inability to carry out the specific oxidation concerned is to be referred to a gene defect. A geneticist with sufficient faith in the versatility of the gene might well suspect this to be the situation. The case does illustrate a point of importance—that man is a most unsatisfactory organism from a genetic standpoint.

All of the examples mentioned above have to do with phenylalanine-tyrosine metabolism in man. Their interrelations are

illustrated schematically in Figure 1. Tyrosine can be oxidized in the 3 position to give 3,4-dihydroxyphenylalanine which in normal individuals is converted to the skin and hair pigment melanin. The structure of melanin is not known with certainty, nor are the reactions by which it is formed. In the recessive genetic trait albinism, some one of these reactions is presumably blocked through a specific gene defect. It is a point of considerable general significance that we would not be able to formulate

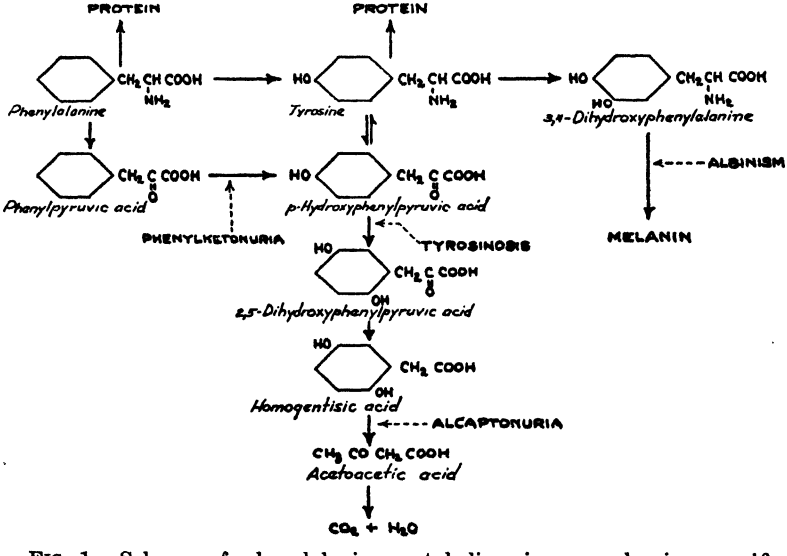

FIG. 1. Scheme of phenylalanine metabolism in man showing specific reactions known to be genetically controlled. Based on an interpretation given by Haldane (5).

the scheme shown in Figure 1 were it not for the indicated heritable metabolic deficiencies. They have been used by the biochemists as tools with which to determine the course of metabolism. It is certain that if more such defective types were available for study, we would understand phenylalanine-tyrosine metabolism in man in more detail and with more confidence. Such considerations as these suggest that significant advances might be made in our understanding of the metabolic processes of organisms in

general if the method of using genes as biochemical tools were applied more deliberately and more systematically. A beginning has already been made in this direction in the study of several organisms and progress is encouraging.

Biochemically as well as genetically, man is an unsatisfactory organism for the type of study indicated. Indeed, it is quite remarkable that we know as much as we do about him. For maximum progress along these lines organisms are needed having life cycles suited to genetic studies and at the same time possessing characteristics that make them amenable to chemical investigation. Various ones meeting these requirements more or less satisfactorily have been investigated by what might be called the method of biochemical genetics. For example, following the pioneer work of Wheldale, the biosynthesis of anthocyanin and related pigments in flowering plants has been studied (6, 7). It is found here too that the action of specific genes can be interpreted in terms of their relation to specific chemical reactions concerned with pigment synthesis and modification. Investigations of a basically similar nature have been made of the eye pigments of insects (7, 8), on the role of carotenoid pigments and their derivatives in sex phenomena in the green flagellate Chlamydomonas (9, 10), of hair pigments in mammals (11), on specific sugar fermentations in yeast (12, 13), and in a number of others (7, 14). In all of these, interpretations on the basis of the gene-enzyme-chemical-reaction concept are possible.

Based on the assumption that genes in general act through controlling protein specificities and consequently enzyme specificities, studies on the red bread mold Neurospora have been undertaken in an attempt to systematically produce mutant types in which particular metabolic defects were present (15, 16, 17). The organism chosen for the study has a short life cycle that is almost ideally suited for genetic study as shown by Dodge (18) and Lindegren (19). It is able to grow on a chemically defined medium containing a carbon and energy source (sucrose for example), nitrate or other inorganic nitrogen, various inorganic salts, and the vitamin biotin (15). From the ingredients of this

medium the organism obviously makes all the compounds for which it finds a need. Since protoplasm is elaborated and since this contains proteins in which most if not all naturally occurring amino acids are present, the organism must carry out all of the reactions necessary for synthesis of these from inorganic materials and sugar. Similarly, since the fungus contains vitamins of the B-group, these must be elaborated by the protoplasm. All other essential compounds with the exception of biotin, which is supplied in the medium, must likewise be synthesized. These include purines, pyrimidines, and many others, some of which presumably are not yet known to us.

If the reactions by which the biologically essential compounds are synthesized are in general under gene control, it should be possible to interrupt particular syntheses by inducing mutations in the genes concerned. Gene mutations are known to be produced in other organisms by ultraviolet light, x-rays, and other radiation, although there is as yet no known way of directing these agents toward specific genes. Mutations are produced more or less at random in response to disturbances produced through ionizations or following absorption of energy in the case of ultraviolet radiation. X-ray or other treatment would therefore be expected to induce mutations more or less at random among the genes concerned with various syntheses.

Neurospora crassa is a heterothallic fungus—that is, there exist strains of two sexes. Each of these reproduces asexually by means of conidia, but sexual reproduction occurs only if strains of opposite sex are grown together. The two sexes are not morphologically distinguishable. The mycelial segments are multinucleate but all nuclei in the vegetative cycle are haploid, i.e., each contains a single set of seven chromosomes. In producing so-called biochemical mutants, conidia of one sex are irradiated and then crossed with a normal strain of the opposite sex. Following the formation of fruiting bodies in which fusion of haploid nuclei from the two parents occurs, meiosis takes place in the conventional manner (20) in spore sacs. The four primary haploid meiotic nuclei undergo mitotic divisions to give eight nuclei

arranged in a row in the spore sac. Each is then included in an ellipsoidal, black, sexual spore known as an ascospore. In attempting to produce mutations affecting specific syntheses, single ascospores descended from crosses in which one parent was irradiated are isolated and grown on a special medium to which is added as many compounds of biological importance as possible. This is done so that a single spore strain carrying a gene defect which prevents synthesis of an essential substance will be able to obtain the substance it cannot synthesize from the medium. Defects in synthesis are detected by transferring the single-spore cultures from the complete medium to one containing only the minimal requirements of the original wild-type strain. Growth on complete medium and failure of growth on minimal medium is taken as evidence of loss of ability to synthesize some substance contained in the complete but not in the minimal medium. What this is can often be determined by making a systematic series of tests on minimal media supplemented with known compounds. On the average, with treatments of conidia with about 50,000 r units of x-ray or 15,000 ergs/mm.^{2} of ultraviolet of wavelength 2536 Å, about one ascospore per hundred gives a culture that grows on complete but fails to grow on minimal medium. The remaining 99 per cent of the spores do not carry mutations or carry changes that do not express themselves in differential growth on the two media. Some of these carry mutations that modify the morphology of the organism.

On analysis of strains in which synthesis is defective, it is found that various compounds are concerned. For example, mutant strains have been obtained in each of which growth is dependent on an external supply of one of the following B vitamins: thiamin, riboflavin, pyridoxin, pantothenic acid, *p*-aminobenzoic acid, nicotinic acid, choline, or inositol (17, 21, 22). It is supposed that in each case synthesis of the particular compound which must be supplied is in some way defective in the mutant strain. In a similar way a series of strains has been obtained in which particular amino acids cannot be elaborated from the materials supplied in the minimal medium (17, 21, 22). Other

strains show still other defects in metabolism such as failure to utilize nitrate nitrogen, inability to grow on certain fatty acids as carbon sources, failure to make pyrimidine and purine compounds (17, 21, 22).

An immediate question is, do these strains differ genetically from the original from which they were obtained? This can be determined readily by the methods of classical genetics. A mutant strain is crossed to a wild-type strain of the opposite sex. After ripe ascospores are produced, sets of eight in their spore sacs are removed from the fruiting bodies. Individual ascospores are taken out in the order in which they occur in the spore sac and planted individually in culture tubes on a medium supplemented with the growth factor required by the defective parent. If the defective strain differs by a single gene or single region of a particular chromosome from the original strain from which it was derived, four spores genetically like each parent are expected in each set of eight. This is because all primary products of meiosis are recovered, each in duplicate. Each spore gives rise to a haploid mycelium which carries at a specific place in a particular chromosome an allele of a gene from either the normal or the mutant type parent. For the decisive gene pair, each spore receives either the normal allele from the normal parent or the mutant allele from the metabolically defective parent. The mechanism is precise and the resulting segregation is therefore a mechanical one of 4 to 4. If all spores are taken from a single spore sac, no sampling error is possible. A further advantage of Neurospora lies in the arrangement of normal and mutant spores which can be of two kinds; either four at one end are like one parent and the remaining four like the other parent, or the two types occur in alternating pairs. Lindegren (19) has shown that the second arrangement results from a physical exchange of two corresponding chromosome segments at the tetrad stage. Thus the frequency of the alternating pair arrangement measures directly the frequency of genetic crossing over between the segregating gene pair and the centromere (point of attachment of the chromosome to the spindle fiber). This, in turn, is a function

of the distance of the gene concerned from the centromere. It is possible, therefore, to determine the location of genes in the chromosomes by observing the relative frequencies of the two types of spore arrangement (19, 21).

When metabolically deficient strains are investigated by the above procedure, the great majority of them prove to differ from the original wild-type in the alteration of one single gene. Occasionally double mutants are obtained that require two substances for growth. In all but one instance, these have proved to have two mutant genes. Their frequency is of the order of magnitude expected on the assumption of independent occurrence of the two mutations, i.e., if one-gene mutations occur in one per cent of the spores, two-gene changes would be expected to occur by chance in 0.01 per cent. The one exceptional case so far encountered involves a mutant strain which differs from the wild type by a single gene but which requires both valine and isoleucine for normal growth (23). It is supposed that these related amino acids have a common step in their synthesis and that it is this step which the gene in question normally controls.

In several instances Neurospora mutant strains have been made use of in studying the course of the biosynthesis of particular compounds. For example, Srb and Horowitz (24) have investigated a series of strains requiring the amino acid arginine or some related compound for normal growth. Seven genetically distinct types have been found. Each is differentiated from the wild type by a single gene but in each of seven strains this distinguishing gene is a different one. This has been established by conventional genetic methods and does not depend on a knowledge of the nature of the defective reactions in the individual strains. Some specific mutant types have occurred two or more times as evidenced by the fact that the seven genetically distinct types were found among fifteen original mutant strains, all of which arose independently. On investigating the nutritional requirements of the seven mutant strains, Srb and Horowitz found that four of them would grow if supplied with minimal medium plus either ornithine, citrulline, or arginine. From this it is deduced that in

these strains the synthesis of ornithine is interrupted. Two strains grow if supplied with citrulline or arginine but not if given ornithine only. They presumably cannot convert ornithine to citrulline. The remaining strain requires arginine for normal growth and will not respond to ornithine or citrulline. It is inferred that it is unable to transform citrulline to arginine. Evidently the sequence ornithine → citrulline → arginine occurs in normal strains of Neurospora. The enzyme arginase is present in this organism and arginine may be hydrolyzed to yield a mole-

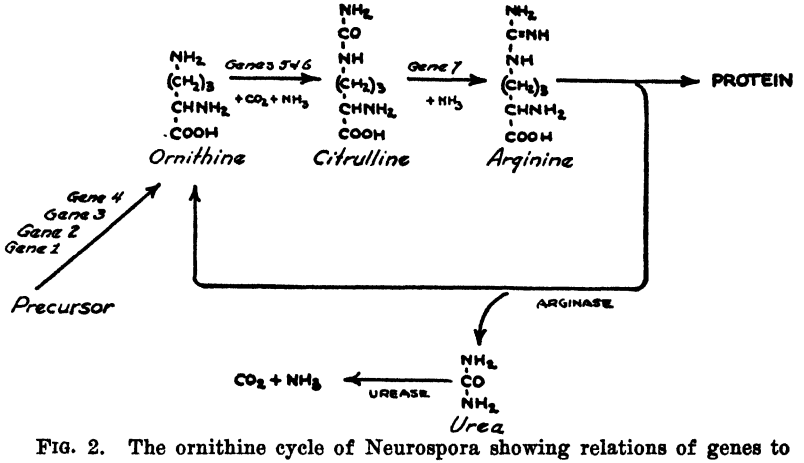

FIG. 2. The ornithine cycle of Neurospora showing relations of genes to particular chemical reactions.

cule of urea and at the same time reconstitute a molecule of ornithine. The urea is further degraded to CO_2 and NH_3 under the influence of the enzyme urease. It therefore appears evident that an ornithine cycle essentially like that postulated by Krebs and Henseleit (25) for the mammalian liver is present in Neurospora (Fig. 2).

It is of interest that in the establishment of the ornithine cycle in Neurospora the use of genetically modified strains of the bread mold has played an essential part. The cycle is perhaps more satisfactorily established in the fungus than in the mammal, and this can be taken as evidence of the usefulness of the genetic

method of determining the course of biosynthesis of a particular compound of biological importance. It is of further interest that the ornithine cycle is found in organisms as unrelated as the rat and the bread mold—in both it appears to follow the same course, even in detail. This suggests that the ability to carry through the ornithine cycle is a basic property of protoplasm.

That genetic defects can be generally useful in working out mechanisms of metabolism in a manner analogous to the use of alcaptonurics in discovering homogentisic acid as an intermediate in the breakdown of phenylalanine and tyrosine in man is evident from the work of Tatum and Bonner on tryptophane synthesis. A number of mutant strains of Neurospora have been obtained that require tryptophane or some related compound for growth (26). It has been found that the original wild-type strain, as well as any of the mutant types so far studied, is able to synthesize tryptophane by condensing indole and serine (26a). This may well be the final step in tryptophane synthesis in all organisms in which this amino acid is made. It was found that a particular mutant strain is unable to synthesize indole as indicated by the fact that it will grow normally only if indole or tryptophane is supplied in the medium. If this strain is grown in the presence of a small amount of tryptophane, it makes and excretes into the medium the compound ortho-aminobenzoic acid (anthranilic acid). A second, genetically different mutant type does not produce anthranilic acid when it is grown under similar conditions, but can be induced to grow normally when it is supplied with this material in the medium. The obvious interpretation is that anthranilic acid is a normal precursor of indole in Neurospora and that in the presence of the one gene in defective form its conversion to indole is blocked. If the second gene is defective, the synthesis of anthranilic acid is itself interrupted (Fig. 3).

Theoretically it should be possible to determine the precursors of anthranilic acid if the necessary mutant strains were available and were studied under the right conditions. There is no inherent reason why the course of biosynthesis cannot be determined by the method of biochemical genetics all the way back to the

inorganic starting materials. We know that the first step in the utilization of nitrate nitrogen through its reduction to nitrite is subject to gene control. Of course it is not possible to determine completely the course of biosynthesis of compounds from inorganic precursors in Neurospora itself because this organism is dependent on an external source of some energy-supplying carbon compound. There is, however, no apparent reason why the same general method cannot be applied in chemo- or photosynthetic organisms.

Using the same general methods illustrated in the above ac-

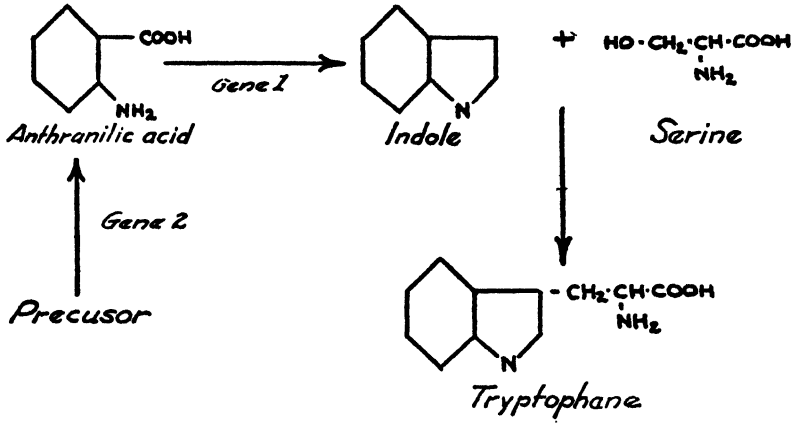

FIG. 3. The course of tryptophane synthesis in Neurospora.

counts of studies in arginine and tryptophane synthesis in Neurospora, investigations are underway which promise to tell us something about the precursors of choline (17) and nicotinic acid (27). In both instances mutant types are known in which the synthesis of the end product is blocked but in which, under conditions permitting some growth, a precursor is produced and accumulated. In each case a second mutant type is known for which the precursor is active in promoting growth. The complete chemical identification of these precursors has not yet been made but the two examples serve to show that the method in which the gene concept plays an important part is applicable in other situations.

The above account indicates that a variety of chemical reactions characteristic of the organism are dependent on the presence of particular genes in a specific form. If these genes undergo mutations of certain types, the specific reactions which they control fail to be carried out in a normal fashion. If the product of a reaction blocked in this way is a stable substance that can be supplied the organism from outside, the genetic and biochemical defect can be circumvented. The organism then becomes dependent on an external source of the particular compound. It is known that evolutionary specialization frequently involves such loss of synthetic ability (28, 29, 30). We ourselves, for example, are dependent on external sources of amino acids, vitamins, and other compounds of biological importance. Presumably somewhere among our remote ancestors were creatures who were more versatile than we in their synthetic abilities. Such studies as those on Neurospora indicate the probable genetic basis of such evolutionary specialization in metabolic function.

A natural and most significant question concerns the nature of the mechanism by which genes are related to chemical reactions. Unfortunately we appear to be a long way from final answers to questions of this kind. It is, however, possible to suggest a hypothesis that may have a use in directing our further efforts to obtain the desired answers. Since this of necessity involves the nature of the gene itself, it is important to consider such evidence as we have in this regard. Direct chemical analysis of chromosomes shows them to be at least largely composed of nucleoproteins of one type or another (31). But since genes may make up only a small fraction of chromatin, this method does not give a final answer. Ultraviolet radiation produces gene mutations and its efficiency per unit energy varies with the wavelength in a manner similar to its absorption by nucleic acid (32, 33). This indicates that the energy effective in producing mutations is absorbed by nucleic acid. The simplest assumption is that this is so because the nucleic acid is a direct gene constituent, but it is of course also possible that the energy absorbed by nucleic acid is transferred to the gene. A third line of evidence, also indirect,

involves a comparison of viruses and genes. Both have the property of self-duplication and in expressing this property they are both dependent on an array of raw materials such as is found in the living cell. On the basis of admittedly crude estimates, genes appear to be of about the same size as medium-sized viruses. Genes are mutable and the process is at least sometimes reversible. This property is likewise characteristic of viruses (34). Such viruses as have been obtained in pure form have been shown by direct chemical analysis to be nucleoproteins (34, 34a, 35). Although many of the larger viruses may be compound structures, corresponding to several or many genes, they are all probably essentially gene-like. The similarity suggests that if one is nucleoprotein in nature, the other probably is too. The total circumstantial evidence that genes are nucleoproteins or contain nucleoproteins is therefore considerable and we are justified in basing a hypothesis on this assumption.

An indispensable property of genes is their power of self-duplication. The thousands of genes present in a single cell must duplicate themselves once every cell generation. If they do contain proteins, their specific proteins must be elaborated before or during duplication. A commonly held view is that gene duplication directly involves protein synthesis (5, 36, 37, 38). The gene may serve as a master molecule or model against which component parts, possibly individually small, of the new gene are put together in a pattern corresponding both chemically and physically to the old gene. An alternative possibility is that proteins are built up stepwise through amino acids or similar compounds, dipeptides, polypeptides, and so on, up to the final molecule, with no step being more complicated chemically than reactions now known to us. Regardless of which of these views is correct—and there seems to be no valid basis for choosing between them at present—the gene can be visualized as directing the final configuration of a protein molecule and thus determining its specificity. A given protein molecule, patterned after a particular gene, might become a component of a new gene like the one from which it was copied or it might become an antigenically active

protein, an enzyme protein, or a storage protein. The factors determining which of these fates befalls a given molecule are beyond our present knowledge. Actually, there is no good basis for supposing that these categories are mutually exclusive.

If genes in some way direct the configurations of protein molecules during their elaboration, it is not necessary to assume that they function in any other way. Their known relations to antigens, which seem usually to be direct (5, 14, 39, 40), would be accounted for if this were so. The protein components of enzymes would likewise have their specificities imposed fairly directly by genes and the one-to-one relation observed to exist between genes and chemical reactions should be a consequence. It should follow, indeed, that every enzymatically catalyzed reaction that goes on in an organism should depend directly on the gene responsible for the specificity of the enzyme concerned. Furthermore, for reasons of economy in the evolutionary process, one might expect that with few exceptions the final specificity of a particular enzyme would be imposed by only one gene.

Whatever is the mechanism by which gene and enzyme are related, there must exist a hierarchy of gene control. On any basis the components from which a given protein molecule is constructed must themselves be synthesized and these reactions, one or more steps removed from the final act of protein elaboration, depend on enzymes which in turn depend on other genes. On this view, the final specificity of an enzyme molecule is set by a particular gene (primary control), but many other genes must play an essential part at earlier stages (secondary control). Experimentally, it is not always easy to distinguish between the primary action of a particular gene and secondary consequences (7). This is only another way of saying that the organism is not merely a bundle of self-duplicating units each acting independently but is rather a most highly integrated and complex system of such units.

It is perhaps unnecessary to state the obvious conclusion that if one is to understand the metabolism of the organism in the most complete way possible, genes must be taken into account.

Too often in the past these units have been regarded as the exclusive property of the geneticist. The biochemist cannot understand what goes on chemically in the organism without considering genes any more than a geneticist can fully appreciate the gene without taking into account what it is and what it does. It is a most unfortunate consequence of human limitations and the inflexible organization of our institutions of higher learning that investigators tend to be forced into laboratories with such labels as "biochemistry" or "genetics." The gene does not recognize the distinction—we should at least minimize it.

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CHEMOTHERAPY OF HYPERTHYROIDISM[1,2]

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LONG before the element iodine was discovered materials now known to contain it were used for the treatment of simple or endemic goiter. During the past thirty years this element which is so effective in the prevention of goiter has been used with success in the treatment of hyperthyroidism. The mechanism of this unexpected effect of iodine is still a mystery though it has been the subject of intensive investigation for many years. The topic of this evening's discussion may seem to be even more curious for it deals with substances which have in common the property of causing goiter and of curing hyperthyroidism.

This review will attempt to show how studies on the mechanism of action of goitrogens led to the concept that certain chemical substances interfere with the normal endocrine function of the thyroid gland and how these compounds can be used for the treatment of hyperthyroidism. For the sake of brevity, and I hope with your permission, reference will be limited to work which has been done during the past four years.

A major stimulus to the recent studies on goitrogens was the discovery by Mackenzie, Mackenzie and McCollum (1) that goiter could be readily induced in rats by the administration of sulfaguanidine. This goitrogenic effect was more rapidly obtained than any previously reported and this was the first chemical substance shown to be goitrogenic in the presence of an adequate or excessive iodine intake. Also during the year 1941 there appeared from a second Baltimore laboratory the report by Richter and Clisby (2) that phenylthiourea is goitrogenic in rats and from Hercus' laboratory in New Zealand reports of studies which

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had extended over several years on the mechanism of the goitrogenic effects of diets containing rape seed (3, 4, 5).

These reports aroused great interest; they provided the basis for extensive studies of the mechanism of action of goitrogenic agents and of the chemical nature of compounds with this interesting property of causing thyroid enlargement. By the end of the year 1941 our interest was diverted from less interesting aspects of thyroid physiology to an investigation of the mechanism of action of sulfaguanidine.

A variety of feeding experiments permitted observation of the food consumption, growth rate and development of young rats given sulfaguanidine. While the thyroid glands of the treated animals were undergoing extraordinary hyperplastic changes characteristic of intense thyrotropic stimulation the animals began to eat less food and eventually to suffer a decreased rate of growth, effects which were reminiscent of those which follow thyroidectomy. It was soon apparent that the treated animals were indeed hypothyroid even though the thyroid glands seemed overactive. Thyroid hormone was then given with the result that the effects of the goitrogen were altogether abolished. This suggested that the goiter was a compensatory change resulting from the induced state of hypothyroidism. When the hypophyses were removed from the experimental animals the goitrogen had no visible effect upon the thyroid gland and even if thyrotropin were injected into the hypophysectomized animals its stimulating effect was not enhanced by the goitrogen given concurrently. Finally, thyroid hormone in various doses up to the lethal level was administered with and without the goitrogenic agent. Neither in normal, in thyroidectomized nor in hypophysectomized animals did the chemical modify the calorigenic or toxic actions of the thyroid hormone.

The conclusion was inescapable that the primary mode of action was an inhibition of the formation of thyroid hormone. Extensive experiments of the Mackenzies led to similar conclusions and the results of Baltimore and Boston investigations were eventually published together in February 1943 (6, 7).

In the meanwhile much more effective compounds were encountered and, as we will see later on, thiouracil proved to be quite suitable for further physiological experiments. Also, the attitude toward these compounds changed—they were no longer considered merely as goitrogens but rather as agents with the specific property of inducing a chemical thyroidectomy. For the want of a more appropriate designation they have been called "antithyroid compounds."

What happens when an antithyroid compound is given continuously to a normal animal in full doses may be illustrated by the effects of thiouracil on the young rat. In Figure 1 it may be

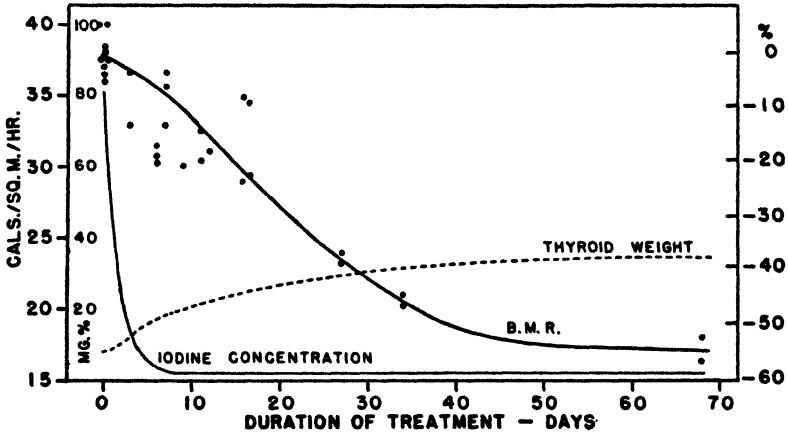


FIG. 1. Effects of the administration of thiouracil in a concentration of 0.1% of the diet to young rats on the iodine concentration and weight of the thyroid gland and on the basal metabolic rate. The scales on the left and right refer to calories per square meter of body surface per hour and the percentage change in metabolic rate respectively. The inner scale on the left refers to the iodine concentration in mg. per 100 gm. of wet tissue and the thyroid weight in mg. per 100 gm. body weight.

seen that the first effect is a nearly complete loss of iodine from the thyroid gland; as soon as treatment is begun no new hormone can be made; one-half of the limited store is used up in one day and all of it is exhausted in five days (8). In the meanwhile the circulating hormone decreases as is manifest by the com-

pensatory enlargement of the thyroid which begins even before the gland is free of hormone and continues at a steadily decreasing rate for several weeks. After a month or so thyroid enlargement just about keeps pace with the growth of the body. The decline in basal metabolic rate lags well behind the exhaustion of the thyroid gland but over the course of two months it progresses to levels which are probably even lower than those which follow thyroidectomy. The curve shown in the figure is a composite one drawn through the scattered points of individual metabolism determinations on different animals. Each animal was used but once in order to avoid the effects of repeated fasting.

Prolonged administration of effective doses of thiouracil to rats causes an arrest of development and a retardation in growth. The younger the animal the more striking the effect and when rats are treated from birth, cretinism results (9). Hughes has also shown that toxic effects of the drug do not contribute to this cretinous condition because growth and development are normal if appropriate doses of thyroxin are given concurrently (10). During the third and fourth weeks of life the mortality among the cretinous animals is very high and it seems logical to assume that a complete absence of thyroid hormone may be incompatible with life in this species; those individuals which do survive may be supposed to have synthesized minimal amounts of thyroxin at times when the concentration of thiouracil in the body may have fallen too low for complete inhibition.

When treatment is initiated later in life the animals are not made really cretinous but growth continues for many weeks. Finally growth ceases but can be reinitiated even after many months by the injection of thyroxin (Fig. 2). Likewise, growth is resumed and may continue for several weeks if thiouracil is removed from the diet for a period of only 24 to 48 hours; suggesting that effective amounts of thyroid hormone can be rapidly synthesized when adequate concentrations of thiouracil are not maintained. In this figure it will also be noted that a small dose of thiouracil given from the 21st day of life for a period of 9½ months resulted in increased growth. This was not just an in-

crease in body weight but actually involved a proportional gain in skeletal dimensions. This might imply that a mild degree of hypothyroidism is conducive to excessive growth in this species, a more reasonable assumption than the postulate that thiouracil in small doses has the property of an accessory food factor.

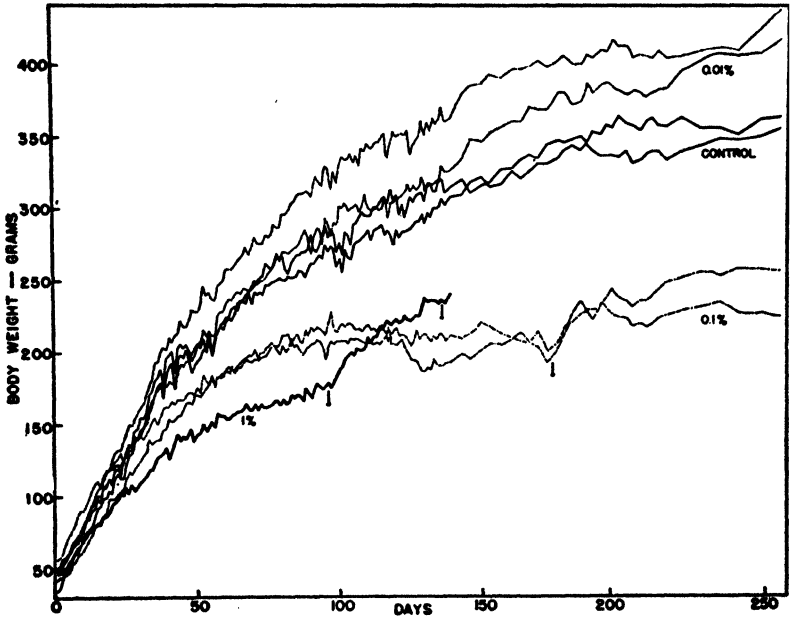


FIG. 2. Body weight changes of normal rats and their litter mates fed diets containing 0.01, 0.1 and 1.0% thiouracil in the diet from the 21st day of life for 255 days. 5 μg. of thyroxin was administered daily to the animal receiving 1.0% during the period indicated by the arrows. A single injection of 100 μg. of thyroxin was given to one of the animals receiving 0.1% at the time shown by the arrow; these two rats were caged together.

The effect of thiouracil in other species and at different ages varies widely both as regards the degree of thyroid hyperplasia and the extent of the changes to be seen in the organism as a whole. The pronounced compensatory hyperplasia which is seen in rapidly growing young rats is much less striking in the adult

animal and still less pronounced in the guinea pig, indeed in very young guinea pigs the thyroid gland may actually be reduced in size by thiouracil. Waldo (11) has found the mouse to be quite insensitive to this compound, even large doses do not induce so great an effect in this species as in the rat. Engle (12) has found that full doses have but a minimal effect in the rhesus monkey—a particularly significant observation in view of the fact that thyroidectomy has so little effect in this species.

The most enormous thyroid enlargement yet seen is that of the thiouracil-treated chick. Also, this species suffers greatly from a lack of thyroid hormone and when full doses of thiouracil are given from the time of hatching a most curious picture results. Body feathers, comb, wattles and spurs remain undeveloped, the muscles are weak, the joints are hypermobile, the body skeleton is much retarded in development but body fat accumulates and the chick finally is unable to stand. This state is presumably the avian counterpart of mammalian myxedema (13).

Some of the lower vertebrates have also been shown to be affected by thiourea and thiouracil. A small tropical fish has been shown not to develop if thiourea is mixed with the aquarium water (14). Normal amphibian metamorphosis is prevented (15) and this has been shown to be due to an effect on the thyroid gland itself, for thyroxin is still capable of inducing metamorphosis in the presence of thiouracil while thyrotropin is not (16). On the other hand the adult newt (*Triturus viridescens*) seemed not to be affected by either thiourea or thiouracil. Singer (17) administered these compounds in the water as well as by injection without observing any effect upon moulting, a process known to require the thyroid hormone (18).

Perhaps the degree of effect to be anticipated in various species and at various ages is best correlated with the intensity of thyroid function which normally obtains. Inhibition of an active thyroid gland would be expected to have more noticeable consequences than the further depression of a gland which is already almost inert. This principle can be demonstrated by merely changing

the environmental temperature. Thyroid hyperplasia in the rat in response to thiouracil can be practically abolished by providing a sufficiently warm environment (19). It will be apparent that such considerations as the foregoing have a bearing on the results to be anticipated from the administration of antithyroid compounds to man.

It would be of considerable biological interest to know whether the action of these compounds is confined to the vertebrates and whether metamorphosis in the lower chordates may not be conditioned by a thyroid hormone. Indeed the possible effects of thiourea derivatives on subvertebrate forms would be an interesting enquiry for perhaps a homologous hormone is important to them also. If it be true that vertebrate tissue other than the thyroid gland can synthesize thyroid hormone, as indicated by the work on thyroidectomized rats by Chapman (20) who used iodine deficient diets and by Morton, Chaikoff, Reinhardt and Anderson (21) using radioactive iodine, then it is quite possible that a thyroid hormone might be synthesized by animals which possess no microscopically recognizable thyroid tissue.

Effects of iodide. The Mackenzies (22) showed clearly that relatively large supplements of iodide did not inhibit the goitrogenic effect of sulfaguanidine. In the case of thiourea it was found that maximal doses of iodide caused only a slight inhibition of thyroid hyperplasia (7). Recently, however, McGinty (23) has shown that added iodide has a marked effect on the iodine content of the thyroids of thiouracil-treated rats. This effect was readily confirmed. The addition of potassium iodide to the drinking water in amounts of from 10 to 1000 mg. per liter inhibited slightly the compensatory enlargement of the thyroid gland resulting from the concomitant administration of thiouracil in the diet for 10 days. The effect on the resulting iodine concentration of the glands was much more striking and was roughly proportional to the dose of iodine (Fig. 3). It was further found that this accumulation of extra iodine did not require ten days, indeed it could occur in a matter of minutes. After 10 days of thiouracil feeding when the thyroid glands are

nearly free of iodine, a single dose of KI caused the iodine concentration to return half way to normal within 15 minutes. In Figure 4 doses of 0.01 to 10 mg. of KI may be seen to cause a proportional increase in iodine in the thyroid gland of thiouracil-treated rats within one hour. The smaller the dose the larger the proportion of the injected dose accumulated. Similarly the iodine-depleted glands of animals fed an iodine-deficient diet rapidly accumulated iodine when KI was given. A full dose of

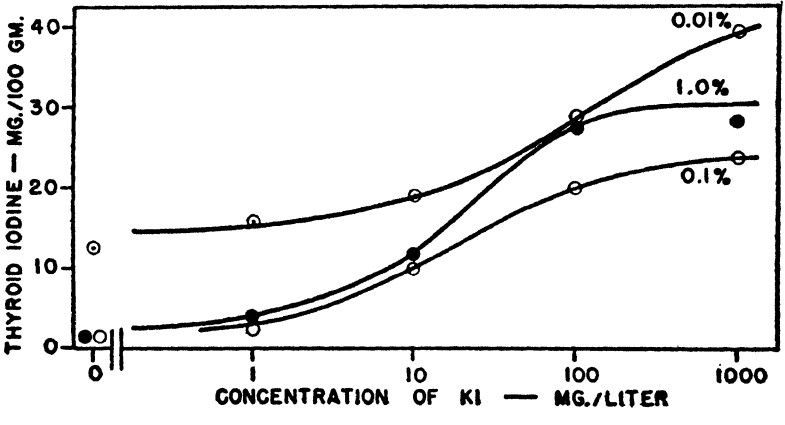


FIG. 3. Effect of addition of potassium iodide to the drinking water in concentrations of from 1 to 1000 mg. per liter (log. scale) on the iodine concentration of the thyroid glands of rats given thiouracil in the diet for 10 days in concentrations of 0.01% (circles with dots), 0.1% (open circles) and 1.0% (solid circles).

thiouracil failed to prevent the immediate uptake of iodine under these conditions also. Such large accumulations of iodine do not follow the injection of KI in the normal animal. The iodine which is rapidly accumulated by the thiouracil-treated gland must enter little if at all into the synthesis of thyroid hormone, for the compensatory enlargement is not markedly modified. The accumulated iodine appears to remain unattached to protein as the experiment shown in Table 1 indicates. The oven-dried glands were finely ground and extracted with distilled water, a procedure which leaves the major portion of the iodine of normal

thyroid tissue with the denatured protein. The iodine newly accumulated in the thiouracil-treated glands was nearly all (96.7%) water-soluble. These findings though obtained by different methods are fully confirmatory to those of McGinty.

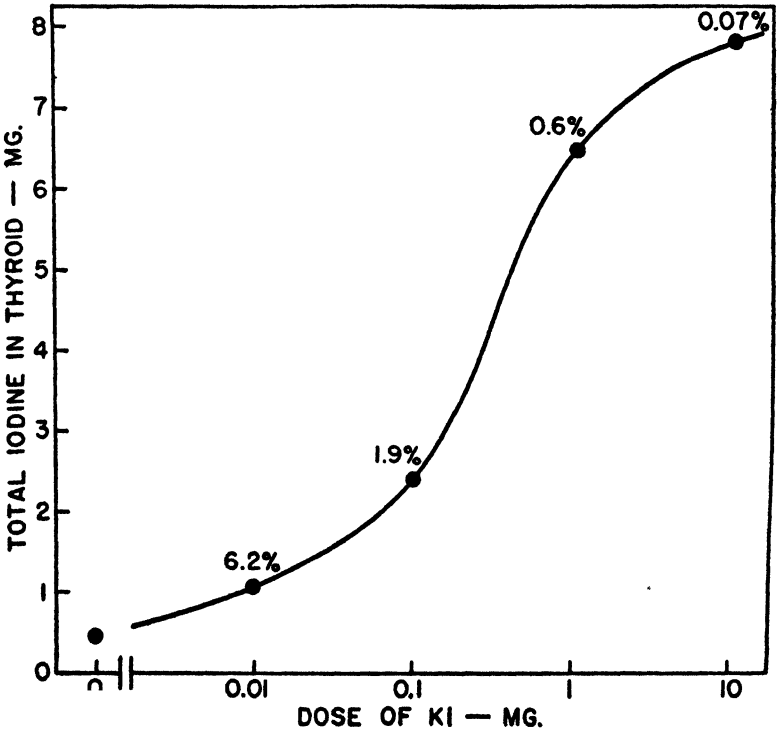


FIG. 4. Effect of a single intraperitoneal injection of potassium iodide in doses of from 0.01 to 10 mg. in 0.1 cc. of water on the total iodine content of the thyroid glands of rats treated for 10 days with 0.2% thiouracil in the diet. The animals were killed one hour after the injection. The figures beside the points represent the percentage of the injected dose which accumulated in the thyroid gland.

A different effect is observable when the dose of thiouracil is only partially inhibitory. When about one-tenth of the fully effective dose of thiouracil is given, thyroid inhibition becomes

TABLE 1

Fractionation of the Iodine Contained in Normal Thyroid Glands and That Accumulated in the Glands of Thiouracil-Treated Rats One Hour after the Injection of 500 μg. of Potassium Iodide

	Total iodine, mg./100 gm. wet tissue	Soluble in water %	Insol. in water %
Normal thyroids	109.8	28.0	72.0
Thiouracil-treated glands after KI	30.8	96.7	3.3

much more marked when the iodine intake is reduced (Table 2). How can these findings be explained? The accumulation of iodine by fully inhibited glands without appreciably influencing the synthesis of thyroid hormone implies that there is a mechanism, distinct from the iodination system, permitting the accumulation and storage of iodide. Once the iodide is oxidized it would presumably be immediately bound to tyrosine and be found in the protein fraction. It is most difficult to imagine what type of combination could exist which would permit iodide to be held in the thyroid.

Iodine deficiency has such a strong potentiating influence on the goitrogenic action of small doses of thiouracil that it is apparent that still smaller doses of thiouracil would be detectable

TABLE 2

Effect of a Low Iodine Diet on the Responsiveness of the Thyroid Gland of the Rat to a Small Dose of Thiouracil for 10 Days

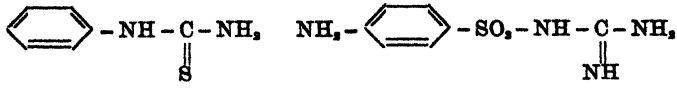
Treatment	Thyroid weight, mg./100 gm. body wt.	Iodine concentration, mg./100 gm. wet tissue
Normal diet	7.8	58.8
Low iodine diet	9.2	39.4
Normal diet + 0.01% thiouracil	12.4	8.4
Low iodine diet + 0.01% thiouracil	31.1	0.9

only if the iodine intake were low. Under such circumstances added iodine would abolish the goitrogenic effect. These considerations reopen the question of the nature of goitrogens which are inhibited by iodine. It has heretofore been assumed that the goitrogenic agent in cabbage and such goitrogens as methyl cyanide and thiocyanate which are effective only in the presence of a low iodine intake are fundamentally different from the thiourea and aminobenzene derivatives (24). This may not be so at all; the apparent difference may be one only of degree, the goitrogens requiring an iodine deficiency may be qualitatively similar but just quantitatively low in inherent potency. This subject requires further study.

The greater effectiveness of thiouracil with low intakes of iodine also has clinical implications. In the treatment of hyperthyroidism a greater effect might be expected in the presence of an iodine deficiency and perhaps smaller doses could be employed if the iodine intake were restricted. Perhaps, too, thyrotoxicosis tends to deplete the store of iodine and it may be that this contributes to the high effectiveness of drugs like thiouracil in hyperthyroidism. Were this true some of the variability in the clinical response to thiouracil could be attributed to differences in the extent of the iodine deficiency at the time treatment is started. This factor could also contribute to the delayed response to thiouracil which is observed in cases previously treated with iodine.

RELATION OF CHEMICAL STRUCTURE TO ANTITHYROID ACTIVITY

By current standards the first goitrogens to be described were of very low activity and the development of highly effective agents was by no means a simple direct process but one marked by many unprofitable detours. The first two compounds to be discovered, sulfaguanidine and phenylthiourea, could be imagined to have several analogous groupings and it was assumed that there was some chemical grouping common



to each which was responsible for their common goitrogenic property. Each can be seen to possess a substituted urea-like group, a benzene ring attached to nitrogen and one sulfur atom. A series of compounds were tested with the aim of finding one which was chemically related to both of these goitrogens. This search was entirely unprofitable and instead it was found that these two compounds represent individual examples of two entirely different groups of active agents. Phenylthiourea is now known to be one representative (and an unusually toxic one) of a class of active compounds having in common the structure

$$\begin{array}{l} =N-C-R \\ \quad\;\; \| \\ \quad\;\; S \end{array}$$

where R is usually a —N= but in selected cases can be an —S— or an —O—. Sulfaguanidine is not active by virtue of its sulfur or its urea-like group but is a member of a series of compounds which owe their activity to the presence of an appropriately substituted aminobenzene grouping.

While this concept was first beginning to emerge the Mackenzies (22) reported the fact that other sulfonamides were active as was thiourea itself; shortly thereafter Kennedy (25) suggested that allylthiourea might be the active goitrogen of rape seed.

At first the problem of the chemical structure responsible for the goitrogenic effect was interesting because it piqued one's curiosity. However, early in 1942 it was already fairly clear that these agents inhibit the formation of thyroid hormone, a concept which suggested that studies on the human being would be most interesting. For this purpose it was naturally desirable to have available a highly active nontoxic compound and so the study of other chemical structures received an added impetus.

The first group of some hundred compounds were tested in rats by a subjective and inexact but nonetheless tedious method. The compound to be tested was given to groups of rats for 10 days admixed with the food or drinking water in various concentrations. The animals were then killed, the thyroid glands inspected for size and evidence of hyperemia and histological preparations of the glands studied under the microscope for evidence of hypertrophy, hyperplasia and colloid depletion.

Though crude this method permitted the selection of thiouracil as a highly active agent suitable for human use (26). A more satisfactory method has been used for the assay of the second and third hundred compounds (8). The substance in question was given to rats as before preferably in the food. At the end of 10 days the thyroid glands were weighed and the thyroid tissue from a group of 3 rats was pooled together for a determination of the total iodine content. Standard curves were constructed relating the daily dose of the drug to the weight and to the iodine concentration in the gland. The responses to various doses of another compound could then be compared with these standard curves and a relative activity could be estimated considering thiouracil to have an arbitrary activity of 1. This method though far superior to the former procedure has serious shortcomings. There is considerable variation in the response to a given dose; a reduction in the iodine content of the diet causes a marked increase in the response; added iodine depresses the weight response slightly while greatly increasing the iodine concentration. These factors are subject to control by the use of large numbers of animals and by running standard doses of thiouracil with each group of substances being tested. A more serious limitation of the method derives from the fact that compounds differ qualitatively and give dose-reponse curves which may differ widely in shape from the standard thiouracil curve. It is obviously impossible to make quantitative comparisons between qualitatively different properties and this difficulty had to be resolved by adopting an arbitrary degree of response and comparing compounds on the basis of the dose required to cause this response. The most appropriate region for comparative purposes seemed to be that which corresponded to a daily dose of thiouracil of 0.2 to 2.0 mg. per 100 gm. of body weight per day. The lower dose had no effect on thyroid size but lowered the iodine concentration to 50% of normal, the higher dose reduced the iodine concentration to one-tenth of normal and caused about a doubling of thyroid size. Even when thus restricted the curves from different compounds did not superimpose and in certain instances a

statement of activity could be little more accurate than a guess. The several curves in Figure 5 are the responses to seven different compounds. The iodine concentration curves are not so unsatisfactory as the weight curves but it is apparent that curve 6

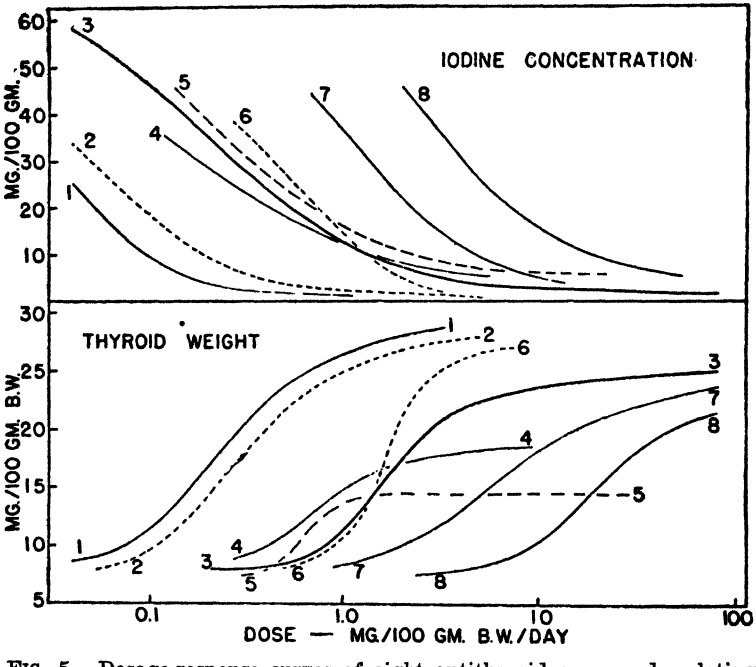


FIG. 5. Dosage-response curves of eight antithyroid compounds relating the iodine concentration (upper curves) and the weight of the thyroid gland (lower curves) after 10 days of treatment to the dose. The dose, plotted on a logarithmic scale, is expressed in mg. per 100 gm. of body weight per day. 1. 6-*n*-Propylthiouracil; 2. 6-Ethylthiouracil; 3. Thiouracil; 4. Thiobarbital; 5. 5,5-Diallylthiobarbituric acid; 6. 2-Mercaptothiazoline; 7. Promizole; 8. Thiourea.

(2-mercaptothiazoline) is steeper than curve 3 (thiouracil standard) while curve 4 (5,5-diethyl-2-thiobarbituric acid) is less steep than the standard curve. The thyroid weight curves differ widely. At one extreme curve 6 (mercaptothiazoline) is very steep while curve 5 (5,5-diallyl-2-thiobarbituric acid) is extraordinarily flat.

At one point curves 3, 5 and 6 cross and at this point, but at no other, the activities of the three compounds could be considered as exactly equivalent. It is obviously impossible to say which is the most active of the three.

Something quite new and fundamental may lie behind these quantitatively unsatisfactory results. A simple matter of rate of absorption and elimination may be the answer to the steepness of curve 6. It has been found that a single dose of 2-mercaptothiazoline has a prolonged action on the thyroid suggesting that it is slowly degraded or excreted by the body. Reasoning by analogy from drugs with this property, this compound could be considered to have a cumulative effect; very small doses may then not give rise to an effective concentration in the body in 10 days and thus give a low response value. With slightly larger doses effective levels would be obtained and then the steady concentration would be highly effective in inhibiting thyroid function. On the other hand no explanation for the flatness of curves like No. 5 have yet been found. It would seem that large doses of compounds such as the thiobarbiturates actively depress the thyroid gland and were this true it would be most interesting to know whether the effect is on the thyroid, on the pituitary or actually on some region of the brain. The sedative effect of the barbiturate is not responsible, for compounds such as phenobarbitol, pentobarbital, chloral hydrate and sulfonal were tested and found not to modify the thyroid response to thiouracil.

For clinical purposes it would be desirable for a compound to have a prolonged action after a single dose so that a steady effect could be maintained by infrequent dosage. This quality is difficult to study but some information can be obtained by comparing the apparent activity of compounds when infrequent doses are given to the test animals. One experiment is shown in Table 3 in which it may be seen that thiouracil is less than one-tenth as effective when injected once daily as when given admixed with the food. Thiobarbital is not quite so quickly disposed of as thiouracil while 2-mercaptothiazoline is just as effective when given every 48 hours as when it is continuously supplied in the diet.

It may be of interest to the chemists present to review briefly the compounds which have recently been tested.

The relative activities of some representative compounds of the thio series are shown in Figure 6. Thiourea is about one-tenth as active as thiouracil and when all of the hydrogens of thiourea are replaced by methyl groups the activity is trebled. This is of interest because of the question of the active form of the thioureylene grouping and suggests that the thio configuration rather

TABLE 3

Influence of the Interval between Doses on the Apparent Activities of Several Compounds

Compound	Relative activity (thiouracil = 1.0)		
	Mixed with diet	Subcutaneous injections once daily	Subcutaneous injections every 2 days
Thiouracil	1.0	0.08	0.03
Thiobarbital	1.7	0.18	0.25
Thiourea	0.1	0.03	0.01
Dithiouracil	1.1	0.07	.....
Mercaptothiazoline	1.3	2.00	1.4

than the mercapto is the state associated with the antithyroid effect. Substitution on the sulfur as in methylisothiourea abolishes activity. The enclosure of a thiourea group into a five-membered heterocyclic ring often enhances activity as well as toxicity; 2-mercaptoimidiazole is more active than thiouracil. Tranposition of the sulfur and one of the nitrogens as in 2-aminothiazole does not abolish activity. It has been found also that one of the nitrogens of the thioureylene group can be replaced by sulfur or oxygen without complete loss of activity as in 2-mercaptothiazoline and 2-mercaptooxazoline. 2-Mercaptotriazole and several of its derivatives are active as are several 2-mercaptothiadiazoles particularly 2-mercapto-5-amino-1,3,4-thiadiazole which is about one and one-half times as active as thiouracil. The only

member of the 2-mercaptothiazine group of appreciable activity is 2-mercapto-5,6-dihydro-1,3,4-thiazine. The three representatives shown of the many inactive compounds of similar constitution, thiopropionamide, methyl-N,N′-dimethyldithiocarbamate and propiorhodanine suggest that there is some further, but as

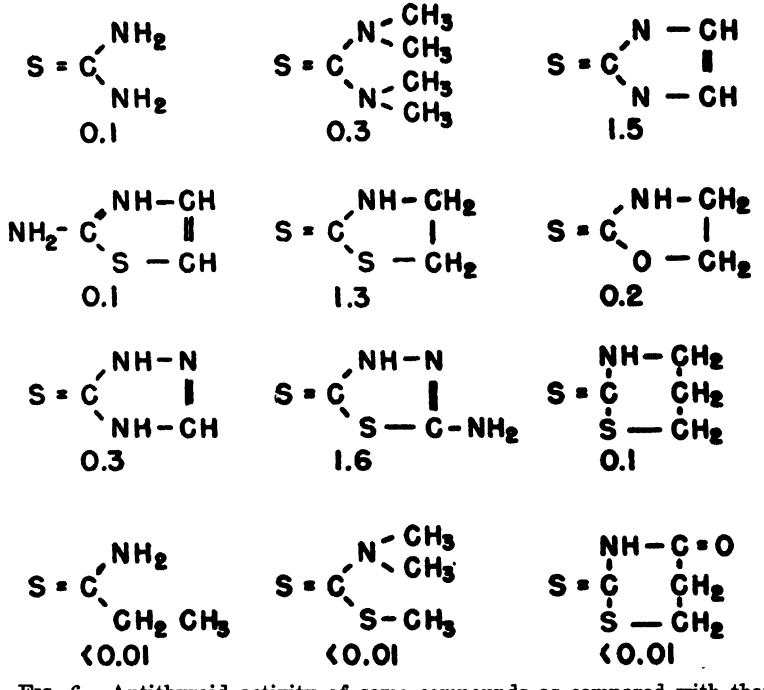


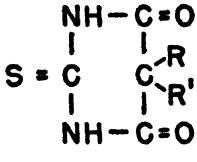
FIG. 6. Antithyroid activity of some compounds as compared with that of thiouracil (activity = 1.00). The compounds shown are: thiourea, tetramethylthiourea, 2-mercaptoimidazole, 2-aminothiazole, 2-mercaptothiazoline, 2-mercaptooxazoline, 2-mercaptothiazole, 2-mercapto-5-amino-1,3,4-thiadiazole, 2-mercapto-5,6-dihydrothiazine, thiopropionamide, methyl-N,N-dimethyldithiocarbamate, and propiorhodanine.

yet obscure, character concerned with biological activity besides the simple nitrogen-carbon-sulfur configuration depicted in the diagrams.

The large series of 2-thiobarbituric acids which have been

tested indicates quite clearly that both of the hydrogens of position 5 must be replaced in order that activity be present (Fig. 7). The dimethyl derivative is about equal to thiourea in potency while the diethyl is well above thiouracil. As larger groups are substituted antithyroid activity falls and the compounds become more potent hypnotics.

Of all the compounds tested to date the most active ones have been found among the alkyl derivatives of thiouracil synthesized recently by Halverstadt, Anderson, Miller and Roblin (27). Substitution on position 5 enhances the effectiveness of thiouracil

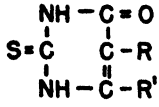


R	R'	ACTIVITY
$-H$	$-H$	<0.003
$-H$	$-CH_2CH_3$	<0.003
$-CH_3$	$-CH_3$	0.1
$-CH_2CH_3$	$-CH_2CH_3$	1.7
$-CH_2CH_3$	$-CH(CH_3)_2$	0.8

FIG. 7. The relative antithyroid activities of some 2-thiobarbituric acids.

somewhat, a maximal increase of three-fold being obtained with an ethyl or isopropyl group. Alkyl groups on position 6 confer even greater potentiation and in this instance also the maximum seem to be near the ethyl derivative which is about seven times as active as thiouracil (Fig. 8). Preliminary toxicity tests in small rats with the limited quantities of these compounds thus far available indicate that toxicity is not proportionately increased.

Curiously enough a number of 5- and 6-substituted thiouracils are completely inert. Groups which thus far have been found to have this unfortunate effect include amino, hydroxy, carboxy, carbethoxy, and cyano groups. The reason for this seeming anomaly is obscure but it would appear that from an analysis of this striking phenomenon may come an answer to the structural basis of antithyroid activity.



R	R'	ACTIVITY
-H	-H	1.
$-CH_2CH_3$	-H	3.
$-CH_2CH_2CH_3$	-H	1.8
-H	$-CH_2CH_3$	7.0
-H	$-CH_2CH_2CH_2CH_3$	3.
-CN	-H	<0.001
$-COOC_2H_5$	-H	<0.003
-H	$-NH_2$	<0.003
-H	-COOH	<0.001
-H	-OH	<0.003

FIG. 8. Effect of various substituents on positions 5 and 6 on the antithyroid activity of thiouracil.

Little of interest has come out of a further study of the aminobenzene series of goitrogens. The only compound found to be of greater potency than sulfadiazine is promizole and the difficulty here is to decide whether the activity should be ascribed

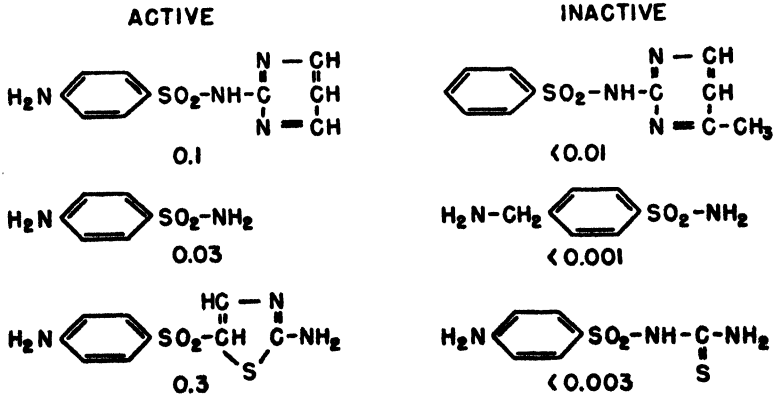


FIG. 9. Three active aminobenzene derivatives and three related compounds of no detectable antithyroid activity.

to the aminobenzene or the aminothiazole portion of the molecule or perhaps to both. In this series all tests to date affirm the necessity that an amino group be attached directly to a benzene ring (Fig. 9).

It is to be recalled that the activities of these compounds have been determined only in one species of animal—the rat, and there is no way of knowing whether comparable relative activities would obtain in man. Thus far only three compounds have been tested in man to a sufficient extent to permit a judgment of their relative activities: thiourea, thiouracil and thiobarbital when compared as accurately as possible in the rat test yield activities in the ratio 0.13, 1.0 and 1.7 respectively. In man doses used for the treatment of hyperthyroidism are about 2, 0.4 and 0.15 grams per day respectively, indicating relative activities of the order of 0.2, 1 and 2.7. Considering the wide error inherent in the estimate of the clinical dosages the values for rat and for man are roughly comparable. This degree of parallelism may not hold for other compounds, however. Two other compounds are known to have been tested in man. Hercus and Purves (28) have reported that 0.4 gm. daily of allylthiourea is effective in hyperthyroidism but that mild toxic effects were so frequently encountered that its use was abandoned. We have tested 6-ethylthiouracil in 12 patients so far. In the dose given no toxic effects have been noted but the minimal effective dose is yet to be determined.

A far greater error in judgment is to be anticipated when one attempts to use animal toxicity studies as an index of the clinical applicability of the newer compounds. The toxic reactions observed in man to thiourea, thiouracil and thiobarbital were wholly unpredictable from animal toxicity tests. Toxic doses of thiouracil in rats give rise to nervous irritability and tremor. In man no neurological disturbances have followed its administration; the common reaction, drug fever, has not been reproduced in animals and the serious reaction, agranulocytosis, has not been observed in animals though a moderate degree of benign leukopenia has subsequently been induced in rats with thiourea

(29). The toxicity of allylthiourea in man could, however, have been predicted from animal studies. It is quite conceivable that a compound which is not highly active in animals and which may even seem to be more toxic than thiouracil might prove to be suitable for human use by virtue of a freedom from the property of provoking sensitivity reactions.

MECHANISM OF ACTION

When the statement is made that these compounds act by inhibiting the synthesis of thyroid hormone all that has been fairly definitely established has been said. To go further and attempt a precise explanation of the chemical processes concerned would involve considerable speculation. The difficulty lies in the fact that the natural process of thyroxin synthesis in the body is poorly understood. One might assume that as starting materials iodide and tyrosine are used. In order that the iodide which reaches the thyroid gland become incorporated into the tyrosine molecule the iodide must be oxidized. The diiodotyrosine must then be subjected to a second oxidation so that two diiodotyrosine molecules might couple through an ether linkage with the loss of one side chain to form thyroxin. It seems likely that free tyrosine is not involved in these reactions but rather tyrosine residues of a protein. This protein might be considered to be part of the enzyme system at first; when some of its tyrosine residues are finally converted to thyroxin residues thyroglobulin results.

While it has been shown by von Mutzenbecher (30) that diiodotyrosine in slightly alkaline solution will spontaneously form a minute amount of thyroxin at a very slow rate, incubation of iodinated casein under somewhat similar conditions yields very large quantities of thyroxin (31). Harrington has shown that oxidizing agents promote the formation of thyroxin from diiodotyrosine *in vitro* (32) and it is probable that free iodine serves as the oxidizing agent for the formation of thyroxin from iodinated casein. This rapid formation of thyroxin *in vitro* from the iodinated tyrosine residues of casein with the formation of a protein containing thyroxin as an integral part of itself may be a model of the process which takes place in the thyroid gland.

For the thyroid gland to effect a similar synthetic process one must postulate an oxidizing system of sufficiently high potential to oxidize iodide. The commonly known biological systems are inadequate for this step and the strongly reducing environment of the cell imposes a further difficulty. Dempsey (33) has described fine granules in the thyroid cell and has presented histochemical evidence that these granules contain a peroxidase which is inhibitable by dilute solutions of thiouracil. Hydrogen peroxide and such an enzyme could effectively oxidize iodide and as Westerfeld and Lowe (34) have shown could also perform the oxidation of the diiodotyrosine to form the diphenyl ether. The difficulty lies in the fact that a peroxidase has not yet been isolated from thyroid tissue by chemical means; Glouck (35) was unable to do so and our many attempts have always failed. In any event at least one and perhaps two enzymes are probably involved in the synthesis of thyroxin and antithyroid compounds could be supposed to act by inhibition of one or the other of these.

A somewhat more likely explanation of the action of the thiourea-like compounds involves their reducing properties. It has been found (36) that thiouracil reacts rapidly with iodine in buffered neutral or alkaline solutions. When one equivalent of iodine is added to thiouracil under these conditions the reaction is almost instantaneous. The iodine is reduced to iodide and the thiouracil is oxidized to the disulfide. At least the disulfide could be isolated in nearly quantitative

$$2\ R-SH + I_2 \rightleftarrows R-S-S-R + 2\ HI$$

yields; it is still possible that an intermediate step was not detected. Thiouracil could be shown to prevent the iodination of tyrosine and of casein by reducing the iodine so rapidly that no iodination was possible. This reaction is not strictly reversible because the disulfide is unstable. However in acid solution the disulfide will oxidize iodide to iodine and thereby be reduced by thiouracil. The point at which the reaction goes half way to completion in the first few seconds is at about pH 2.5 and this implies that the redox potential of the thiouracil-disulfide system is less than three hundred millivolts negative to the iodine-iodide

system under physiological conditions. Most of the thio compounds tested will reduce, at a progressively decreasing rate, several equivalents of iodine. As eight equivalents appears to be maximal it is possible that the sulfur is oxidized to sulfate. If such a reaction were postulated to take place within the thyroid cell it would effectively explain the observed defect in iodination for any oxidation of iodide would be, in effect, prevented. Such a theory must take into account the fact that there are many compounds containing a sulfhydryl group such as cysteine and glutathione which are equally efficient in reducing iodine but which are without effect upon the thyroid gland. This difficulty could be resolved if one assumed that the special system of high oxidation potential which effects iodide oxidation is shut off from the reducing environment of the cell, perhaps in the tiny granules described by Dempsey. It would then be possible that compounds such as cysteine would be oxidized before they could penetrate into this special area, and only those compounds whose redox potential is sufficiently high could survive in the reduced state to reach the enzyme. It is perhaps significant that all the active compounds in this series thus far tested react rapidly with iodine.

This theory has the added disadvantage of not accounting for the action of compounds of the amino-benzene type. From the *in vivo* studies heretofore mentioned thyroxin synthesis seems to be affected at the first step, i.e., the oxidation of iodide. Both classes of antithyroid compounds could be supposed to inhibit the enzyme concerned; the thio compounds could prevent this step by virtue of their reducing properties. Neither class of compounds seems to interfere with the curious mechanism which permits the thyroid gland to accumulate iodide.

In vivo studies using radioactive iodide would seem to show that these antithyroid agents markedly inhibit the accumulation of iodine in the thyroid gland (37, 38, 39, 40), findings which are at variance with the rapid and extensive accumulation of iodine referred to heretofore which follows the injection of large doses of iodide into thiouracil-treated rats. This disparity can only partially be resolved on the basis of differences in the dose of

iodide. It is of great interest in this connection that *in vitro* experiments with thyroid slices indicate that neither the sulfonamides (41) nor thiourea derivatives (42) inhibit the uptake and concentration of iodine by thyroid tissue, although these compounds markedly inhibit the incorporation of this iodine into diiodotyrosine and thyroxin. The latter findings support the view that the inhibition of thyroid hormone formation by antithyroid compounds involves an early step in the synthetic process. Miller and others (43) have recently shown that radioactive iodide exchanges rapidly *in vitro* with the normal iodine in diiodotyrosine under certain circumstances. Whether a similar exchange can occur *in vivo* has not been determined and the extent to which this discovery should modify the interpretation of the studies with radioactive iodine is still an open question.

TREATMENT OF TOXIC GOITER

Just three years ago this month the first clinical trials in hyperthyroidism were initiated. Thiourea was used first; it seemed to be the safest of the active compounds known at that time. In our experiments on rats it was particularly non-toxic—rats could not be killed by any dose which was mechanically feasible to introduce into the animals. It was perhaps fortunate that it was not then known that in certain colonies thiourea is highly toxic to adult rats. The Mackenzies (44) and Richter (45) subsequently found that in their animals a few milligrams was lethal within a few hours, death being accompanied by pulmonary edema and pleural effusion. This is a most interesting phenomenon in itself for the low toxicity of thiourea has been attested by thorough studies on rats, mice, guinea pigs, rabbits and dogs (46, 47). Still more puzzling is the recent report of Greisbach, Kennedy and Purves (48) that rats are rendered unsusceptible to this high sentitivity to thiourea by pretreatment with potassium iodide. In any case all this came later and it was with not too great misgivings that a few grams daily of thiourea were given to the first human subjects.

The first tests were completely negative and it was only much

later that it was found out why—treatment was not continued for long enough. The first cases had had iodine and it was not then realized that in such instances the response is slow.

Finally in July 1942 a previously untreated case of Graves' disease was treated with 1 and later 2 gm. of thiourea daily. By the end of two weeks the B.M.R. had fallen to normal and nearly all symptoms had abated. Though definitely effective thiourea had a short life as an experimental compound for shortly thereafter thiouracil had proven its much greater effectiveness and in September replaced thiourea in the clinical work (49). It is now less than two years since the first report on the effectiveness of thiourea and thiouracil, but in this brief interval reports on more than 700 cases treated with thiouracil have appeared in the medical literature; only about 70 cases treated with thiourea have been recorded.

The response of individuals with thyrotoxicosis to drugs like thiouracil can most readily be discussed from the three aspects of the disease which make up the clinical picture, i.e., the hyperthyroidism, the ocular phenomena and the goiter.

The hyperthyroidism. The excessive quantities of thyroid hormone secreted into the blood stream come from overactive thyroid tissue which contains very little hormone within itself. In Graves' disease this tissue makes up the major portion or all of the thyroid gland, while in toxic adenomatous goiter there is always a certain variable amount of uninvolved thyroid tissue which presumably is not depleted of hormone. Associated with this difference and perhaps because of it, the responses of the two clinical types of hyperthyroidism to antithyroid therapy may differ. When hormone synthesis is inhibited the thyroid gland of typical Graves' disease is quickly depleted of hormone and the hyperthyroidism is rapidly relieved. The rate of response in toxic adenomatous goiter is more variable; a longer delay in response denotes a larger store of mobilizable thyroid hormone. The greatest delay of all is observed in patients with Graves' disease who have been treated with iodine for long periods of time and who have failed to be controlled by this form

of treatment. In them the thyroid gland is overfilled with hormone and presumably all of it must first be utilized before a metabolic response to antithyroid therapy becomes manifest. When treatment is given to an individual with a normal thyroid

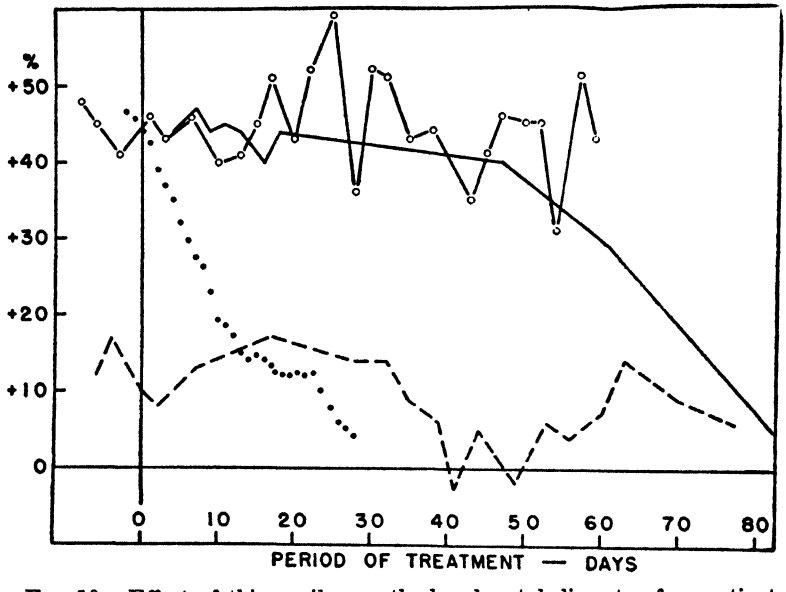


FIG. 10. Effect of thiouracil upon the basal metabolic rate of: a patient with Graves' disease who had been treated for the previous 18 months with iodine—this is the slowest response to thiouracil encountered so far (solid line); a patient with a large nodular goiter who received full doses of iodine prior to and during the period of thiouracil treatment to which there was no response in 59 days (open circles); a patient with advanced congestive heart failure with no symptoms or signs of thyroid disease who showed no effect from thiouracil within 77 days (dashed line). The individual dots describe the average rate of response of previously untreated cases of Graves' disease to thiouracil (60).

gland the latent period is even longer for although the store may not be so large, it is utilized at a slower rate. In Figure 10 one of the slowest responses thus far observed, that of a previously "iodine refractory" case of Graves' disease, is compared with the average rate of response of previously untreated cases of

Graves' disease. Also shown is a case of advanced congestive heart failure without evidence of thyroid disease which showed no metabolic response to thiouracil in two months and a case of adenomatous goiter. This last case had had full doses of iodine for many months previously and continued to receive full doses during the period of thiouracil treatment. Cases such as this one suggest that continued iodine therapy may reduce the effectiveness of thiouracil, an hypothesis in keeping with the findings in animals which have already been mentioned.

Aside from differences in rate the response of patients with hyperthyroidism is quite uniform. All of the manifestations associated with excessive amounts of thyroid hormone disappear. Sloan and Shorr (50) have demonstrated that all of the metabolic and biochemical abnormalities commonly associated with the disease are also corrected in parallel with the restoration of normal metabolic levels. Measurements of the circulating protein-bound iodine (51) indicate that the level of thyroid hormone in the blood falls a little more quickly than the basal metabolic rate, as one might have expected.

Once initiated, the metabolic response continues in a regular fashion and if the usual therapeutic dosage is continued too long a state of early myxedema will eventually be reached and it is common practice to permit a near approach to myxedema before the dosage is reduced. Certainly the early manifestations of hypothyroidism are the most convincing signs that the drug is being given in an effective dose and this state has served as an end point in the evaluation of the potency of new agents. While a short period of hypometabolism may be advisable in some patients such as those with heart failure, marked emaciation or extensive muscular wasting, it is probably best avoided in most cases if possible. Early myxedema has been so frequently observed by those who have used these drugs extensively that it has become readily recognizable in the facial expression. On further investigations one can confirm his impression in the slowed cerebration and speech, the dry and puffy skin and the lowered heat production and so forth but most characteristic of all is the rapid en-

largement of the thyroid gland. Shorr (52) has found that the rising concentration of the serum cholesterol is a useful index of the approach of subnormal metabolic levels.

The ocular phenomena. Judging from simple clinical observation the common ocular changes of exophthalmic goiter seem to improve along with weight gain and increased muscular strength. Lessening of the lid retraction and a decrease in the width of the palpebral fissure makes it appear that the degree of exophthalmos is lessened. Careful measurements of the actual proptosis have not yet been published but Dobyns (53) has shown that as hyperthyroidism is controlled, whether by thiouracil, iodine or thyroidetcomy there is initially a measurable increase in the forward displacement of the eyes. Likewise, thyroid therapy in myxedema causes a slight but detectable recession of the eyeball. One might cite this as evidence that the thyrotropic hormone is concerned in the etiology of exophthalmos, but at the same time it is to be recalled that the retrobulbar contents take part in some of the same changes as other tissues. Perhaps muscle mass and fat are restored here as elsewhere when Graves' disease is controlled and perhaps fluid accumulates here in myxedema as it does in other parts of the body. The eyes are pressed forward as obesity advances and they sink deeply into their sockets in advanced starvation. As far as clinical observations go there seems to be no specific or consistent effect of thiouracil on the exophthalmos of Graves' disease beyond the fact that the appearance of the eyes improves progressively when the metabolic abnormalities of the disease are controlled.

This is not the time to discuss progressive or malignant exophthalmos. Mulvany (54) has presented a forceful argument that this is a different disease and, as far as we know, thiouracil has not been shown to be associated with its cause or cure.

The goiter. Most observers agree that little change occurs in the size of the thyroid gland during the initial period of antithyroid treatment. Sometimes the gland may seem to be a little larger at first and later to decrease in size but more often it becomes progressively less prominent. Clinical observations are

made difficult by changes in consistency—a hard gland seems larger than a soft one, but in many hundred published cases there has been no record of a substantial increase in the size of the gland during the first month or so of treatment. On the other hand Rawson *et al.* (55) have compared the histological pictures before and after a short period of thiouracil treatment preparatory for thyroidectomy and have reported that in 2 of 5 cases the size of the acinar cells increased significantly during treatment. It is a common observation of surgical clinics that, following thiouracil, thyroidectomy is more difficult than following the usual iodine therapy because of the greater vascularity and softness of the gland in the former case (56, 57, 58, 59). It is quite likely than in 20 years surgeons have forgotten what a non-iodinized thyroid gland looks like and we have no way of knowing whether thiouracil treatment actually causes any increased vascularity under these circumstances. The clinical evidence of increased vascularity detected by feeling or listening over the thyroid region seems not to be influenced at first by thiouracil. Graves' disease is known to be subject to fluctuation and it remains to be proved that thiouracil treatment causes a change in the thyroid gland during the interval when the metabolic rate is falling to normal. A very different situation obtains when the metabolic rate is depressed below normal; a rapid and sometimes a very striking enlargement of the thyroid gland occurs under such circumstances, and in a few cases evidence of increased vascularity appears. This enlargement is a common observation when an antithyroid drug is continued in excessive dosage and it has been a consistent finding in the few experiments on individuals with initially normal thyroid glands. Indeed thyroid enlargement has come to be one of the most characteristic signs of excessive dosage. It is also interesting that these enlarged, hyperemic glands still show the response to iodine which is characteristic of the hyperplastic thyroid of Graves' disease (60). When adjustments are made in the dose so that a normal metabolic rate is maintained, no appreciable enlargement of the thyroid gland is observed, and after several months the gland

may actually become appreciably smaller. Finally when the stage of remission is reached regression of the goiter is common.

We are therefore faced with the anomaly that very little thyroid enlargement results when the elevated metabolism of the thyrotoxic patient is brought to normal and maintained there for long periods of time while very marked enlargement ensues if early myxedema is induced with an antithyroid compound in either the thyrotoxic or normal individual. This implies that inhibition of the overactivity of the gland is no stimulus to thyrotropin production while depression of thyroid function below the normal does provide such a stimulus. Can we reason then that an excessive thyrotropic stimulus is not causally related to Graves' disease? Perhaps we can. We have the further observation that in certain individuals a thyrotoxic state antedated specific therapy by a year or more and yet thyroid enlargement was minimal. In some of these a rapid enlargement occurred when the metabolism was depressed too low by therapy. If rapid enlargement is the expected consequence of an overproduction of thyrotropin then Graves' disease must have some other basis.

This same line of reasoning can be applied, though with less conviction, to the problem of the etiology of exophthalmos. Were it true, as it is widely held, that the thyrotropic hormone is concerned with the etiology of this condition one would expect exophthalmos to be a frequent complication of simple non-toxic goiter rather than a concomitant of hyperthyroidism. It is recognized that exophthalmos is very rarely associated with myxedema (61) and is almost unknown as a sequel to total thyroidectomy in individuals with normal thyroid function, conditions which would be expected to be associated with maximal thyrotropin production.

REMISSIONS IN GRAVES' DISEASE

Some of the earlier patients having taken thiouracil daily for many months became tired of their pills and decided that they didn't need medicines any longer. Surprisingly enough they remained well—one of them for two years now. This accident suggested that the drug might well be withdrawn from other

patients and it was arbitrarily decided that 6 to 9 months might be a sufficiently long period of treatment. In Figure 11 are listed all of the patients to date who have been given extended periods of adequate therapy and it is somewhat surprising that this arbitrary management has been so consistently successful. At the top of the figure are 6 cases who relapsed after a first course of

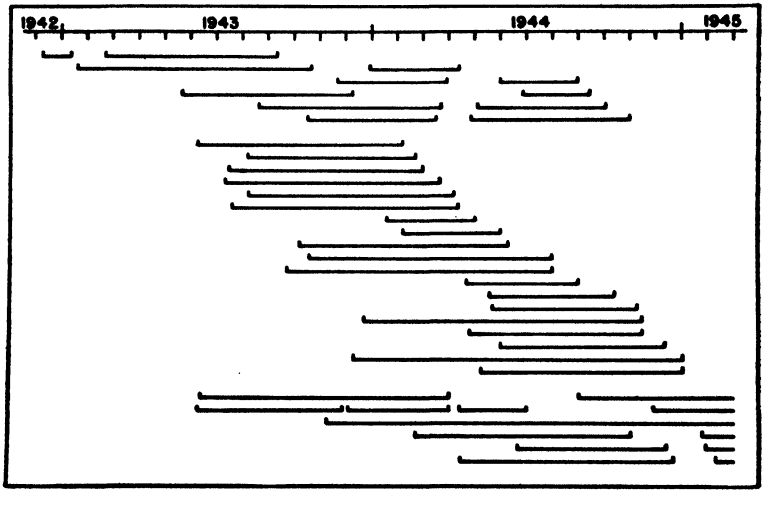


FIG. 11. Summary of all cases treated continuously with thiouracil or thiobarbital for several months. The lines show the periods of treatment, the intervals between the end of the lines and the edge of the chart are the periods during which the patients have remained well without treatment. The first 6 cases are ones which required a second course of therapy before a sustained remission occurred, the next 19 are patients who have now been well without treatment for 2 to 12 months after a single course, and the last 6 are individuals who still require medication.

treatment only to undergo a prolonged remission after a second course. Then are listed 19 cases who have apparently experienced a remission after a single course and who have now remained well for periods of time varying from 2 to 13 months. The last 6 cases are still failures as far as lasting effects are concerned and of these 3 seem to be really refractory—the other 3 have not yet had a fair chance.

It would be most helpful if there were some way of knowing when treatment can safely be discontinued. The best that can be done at present is to maintain a state of normal health for an arbitrary period of time and then to reduce the dose to a level which is below the usually effective level, e.g., 100 mg. of thiouracil once daily. If after 6 to 8 weeks there is no evidence of a relapse the treatment can usually be discontinued. The period of time that the patient is well may be more important than the total duration of treatment. An illustration of this is given in Figure 12 which shows the course of treatment in an iodine pre-

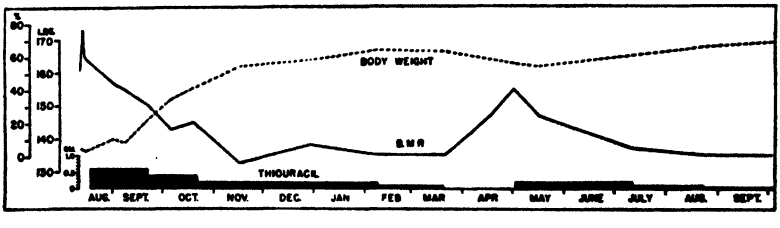


FIG. 12. A slow response to thiouracil in a 54-year-old man with severe Graves' disease who had received iodine for eight months previously. When thiouracil was discontinued after 7 months of treatment the metabolic rate rose and there was some weight loss. After 5 months of further treatment a sustained remission occurred.

treated case. Although the first course of treatment extended over 7 months, the patient was well for only 4 of these because of the slow response. When thiouracil was discontinued hyperthyroidism promptly returned but a second 5 months' course of treatment was followed by a remission.

The course of the patient shown in Figure 13 shows that spontaneous remissions do occur in this disease. This 35-year-old Italian woman entered the hospital with a typical history of Graves' disease and was found to have most of the classical signs associated with that disorder. Iodine was given in preparation for thyroidectomy and as shown in the chart there was no response to it. The patient finally volunteered the information that her disease was caused by her husband's attachment to his

mother; whenever the patient had a disagreement with him he would leave her and return home to his mother. Each time this happened the patient would suffer the same symptoms for which she had entered the hospital. During her hospital stay reconciliation was effected, and the patient announced that she would now recover and refused to have an operation performed. Thiouracil was to be given but through a misunderstanding the patient returned home with no medication. Subsequent events bore out

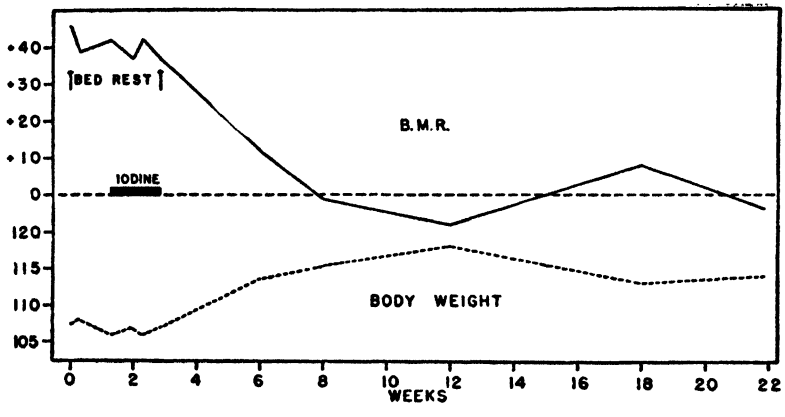


FIG. 13. Changes in the basal metabolic rate and body weight of a patient with Graves' disease. There was no apparent effect from 3 weeks of bed rest and 12 days of treatment with full doses of iodine. A spontaneous remission followed an adjustment of domestic difficulties. See text.

the patient's prediction—she recovered completely and the thyroid gland decreased in size in a striking manner. Aside from one brief recurrence of the family difficulty which was reflected in a mild recurrence of symptoms and signs of Graves' disease, an elevation of the basal metabolic rate, and a loss of weight the patient has remained well.

This is not to imply that all of the cases treated with an antithyroid drug would have recovered without it. However it does indicate that some of them may have improved without specific therapy and that sustained remissions can occur spontaneously.

The results of continued thiouracil therapy have not yet been

recorded from many clinics. Rose and McConnell (62) have observed 8 cases of lasting remission out of a series of 37 which were treated for short periods of time with thiouracil, and Williams has had a similar experience (63).

In a year or two these cases will be most instructive and already an analysis of them indicates that this form of therapy is going to compete with the established custom of thyroid ablation. In favor of chemotherapy rather than surgery are the facts that the metabolic rate can be maintained at any desired level, that no really refractory cases seem to occur, that the drug continues to be effective no matter how long it is given and no matter how often it must be readministered. The high incidence of lasting remissions compares favorably with the incidence of remission after surgery. Thompson's careful analyses of the results of thyroidectomy as performed in two large thyroid clinics showed that 19.5% and 17.5% of cases suffered relapse or persistence of the disease after operation (64, 65). Heretofore the majority of these uncured cases have had to be treated by nonspecific medical means for many years, while some have had to undergo repeated operations.

The only explanation for the lasting effects of a course of thiouracil therapy would seem to lie in the inherent tendency of the ill person to recover. The antithyroid treatment cannot be supposed to do more than to sustain life, an essential factor, and to provide a period of good health, a contributing factor, to spontaneous recovery.

TOXIC EFFECTS

Although not the most common side effect, agranulocytosis is the most serious complication of the use of thiouracil in human beings. It seems to be most commonly seen after a month of treatment and to become less frequent as the period of treatment is prolonged. It has not been reported to occur later than 4 months from the start of treatment. It is to be distinguished from a benign leukopenia which commonly is seen earlier in the course of treatment when, without symptoms of any kind, the leukocyte count falls slowly over a period of several days only

to rise again to normal whether the drug is withdrawn or not. As the total leukocyte count can fall to as low as 2,000 with 12 to 15% of granulocytes in this benign condition it is understandable that the two conditions have been confused. In agranulocytosis the circulating granulocytes disappear rapidly and practically completely and there is usually a severe constitutional reaction and high fever. This event is usually preceded by symptoms such as those of fever or pharyngitis and a diagnosis of grippe is suggested. Sometimes a minor surgical operation, an infection or the administration of some other drug has preceded the agranulocytosis and may have been contributory to it. These two forms of granulocytopenia represent two typical responses and there are doubtless variants which fall somewhere between.

The common reaction—drug fever, sometimes associated with a skin rash is not a serious condition but usually it is impossible to readminster thiouracil and another drug must be substituted. A variety of other side effects have been described in the medical literature but it is most difficult to decide which are caused by the drug. These have included jaundice, edema with elevation of the serum chloride concentration, transient swelling of the submaxillary glands, lymphadenopathy and gastrointestinal disturbances. It would be easier to form an opinion on these complications if their relationship to thiouracil had been established by readministration of the drug.

Those who favor the use of thiouracil as a preoperative measure claim that it is superior to the usual iodine therapy because the hyperthyroidism can be completely controlled before the operation is undertaken. The reason for subjecting the patient to surgery after the disease has been completely controlled is not quite so clear; the reason most often cited is the danger of toxic reactions. As the period of treatment required to obtain an optimal state of health before surgery is a month at least and may be several months in cases which have received iodine, these patients have already been exposed to the hazards of toxic drug reactions, and they are operated upon after the most dangerous period is past. They are then subjected to all of the dangers

of surgery. While some of the best surgical clinics report a mortality rate from thyroidectomy in Graves' disease of about 1% the rate for the country as a whole is probably much higher and has been as high as 13% even in large institutions (66). The little that is known of the over-all risk of antithyroid therapy compares very favorably with the best of surgical results.[3]

CLINICAL USE OF NEWER COMPOUNDS

The side effects encountered with thiouracil have prompted a study of the action of other compounds in man; while these studies are still far from complete a brief review of the findings to date might be of interest.

Thiobarbital. This substance is so closely related to the familiar barbital or veronal which has been used so widely in clinical medicine that it seemed an appropriate compound for clinical trial. At first it was given in a total daily dose of 0.2 to 0.4 gm. Several patients were made drowsy by the larger amount. A few cases were given an initial dose of 0.5 to 1.0 gm. during the first day and then a smaller dose thereafter. These dosages were uniformly effective (Fig. 14) in the control of the disease but were attended by a high incidence (7 of 34 cases) of a peculiar form of febrile reaction. The fever usually appeared during the second week of treatment, was accompanied by malaise and pains in the muscles or joints, and lasted for 4 to 7 days even when the drug was immediately withdrawn. When the drug was resumed after defervescence the fever did not return and in two instances the fever abated while the drug was being continued. In addition to these 7 cases several patients complained of vague symptoms during the first three weeks of treat-

[3] An incomplete review of the medical literature in March 1945 disclosed 731 cases treated with thiouracil. Eleven of these are reported to have developed agranulocytosis (1.5%) and three to have died (0.4%). Many of the reports are quite incomplete and in several instances the toxic reaction alone was reported without any statement of the total number of cases treated. Only 59 records of patients treated with thiourea could be found and among these there was one instance of agranulocytosis and one of leukopenia with thrombocytopenia.

ment and while there was no fever these symptoms may also have been due to a similar but milder reaction. Two fatal complications were encountered in this small series of patients, one of acute hepatic failure and one of agranulocytosis. In both cases the clinical course was complicated by many other factors, and it cannot be determined whether the drug was wholly or partially responsible.

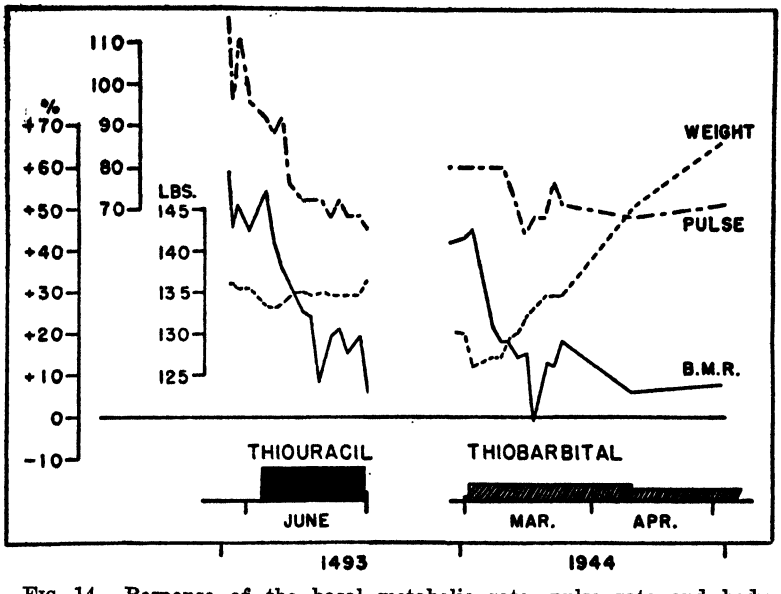


FIG. 14. Response of the basal metabolic rate, pulse rate and body weight to thiouracil (0.6 gm. daily) and, after an interval of 8 months of inadequate and intermittent thiouracil therapy, the response to thiobarbital in a dose of 0.2 gm. daily. The patient, a man 60 years old, was an instance of moderately severe Graves' disease.

Later on, when the earlier cases had been under treatment for several months, it became apparent that excessively high doses were being used; several cases on maintenance doses of only 100 mg. once daily developed early myxedema. Quite recently several cases have been treated from the start with 100 mg. daily in either one or two doses and all have responded well. Apparently then

thiobarbital is not only more active than thiouracil but the effectiveness of a single dose is more prolonged (67).

It remains to be seen whether the high incidence of toxic reactions encountered in the earlier cases was due to an excessive dose and a cumulative effect. If the smaller dose is consistently effective this compound should be of clinical use.

6-ethylthiouracil. To date only 12 cases have been treated with this compound and as the dosage used has not always been effective but has varied from 20 to 100 mg. daily little significance can be attached to the fact that no side effects have been observed. The minimal dose which is consistently effective has not yet been determined but it is already apparent that this compound and certain other 6 substituted thiouracils are highly active in man (68).

CONCLUSION

The foregoing considerations should not leave the impression that the endocrine function of the thyroid gland is now a well-understood process. The little that has been learned about this gland merely reveals new mysteries and we are left with as many questions as before. The fundamental basis of hyperthyroidism is quite obscure; there is evidence to suggest that either the higher centers of the brain, or the anterior lobe of the hypophysis or even the thyroid gland itself could be the site of the primary disturbance. Then, too, there are two forms of the disease; the etiology of toxic adenomatous goiter is probably not the same as the cause of Graves' disease. It would be of great interest to know something about the mechanism whereby large doses of iodine bring about a regression of the hyperplastic thyroid gland of Graves' disease and a relief of thyrotoxicosis. Is this phenomenon related to that property of thyroid tissue which permits it to accumulate iodine so selectively and then by a second mechanism to convert it into a suitable form for its incorporation into the active hormone? Or does iodine have a further property of acting upon some unknown pathological process which is etiologically related to Graves' disease?

It is not too hard to imagine that the mode of action of antithyroid compounds will soon be elucidated. This implies that further information on the normal process of thyroxin synthesis and on the metabolism of iodine will also be obtained. Other questions seem now to be more difficult to answer; the cause of exophthalmos, its relation to Graves' disease, and its occurrence in two apparently distinct forms are problems which seem as far from a solution as ever.

From purely practical considerations it would seem important to learn more about naturally occurring goitrogens and thus to find ways of preventing and treating endemic goiter. The provision of small amounts of iodine to the general population has decreased greatly the incidence of goiter but has not, by far, eradicated the disease. Prevention of goiter would decrease the incidence of the more serious diseases of the thyroid gland. When means are found to prevent the occurrence of toxic reactions from antithyroid drugs or when a compound is discovered which does not give rise to side effects then it will be possible to treat all cases of hyperthyroidism by medical means. The prevention of simple goiter should largely obviate surgical measures directed to the removal of deforming or dangerous thyroid enlargements and so it may not be long before the common practice of partial thyroid ablation will pass into history.

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RESPIRATORY CONDITIONS IN THE FETUS AND EFFECTS OF THEIR IMPAIRMENT[1]

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I SHALL propose this *Probleme* to the Learned; namely, How the *Embryo* doth subsist after the *seventh moneth* in his *Mothers womb?* when yet in case he were borne, he would instantly breath: nay he could not continue one small hour without it? and yet remaining in the *womb,* though he pass the *ninth moneth,* he lives and is safe without the help of *Respiration.*"

So wrote William Harvey (1) almost three centuries ago. The mysteries of intrauterine respiration and the conditions that make possible the change from this to pulmonary breathing at birth have intrigued biologists ever since. Though much progress has been slowly made since Harvey's day, not all the facts are in, not all discoveries known.

What are the mechanisms by which the fetus obtains oxygen, what measures of safety are provided against the possibility of asphyxiation in utero, and what are the effects of their failure?

The placenta is the respiratory organ of the fetus. One can picture its circulation rather simply. A stream of well-oxygenated maternal blood enters the placenta and comes into intimate contact with the fetal blood stream, poor in oxygen. The two do not mix, but the degree of contact differs in various species of mammals and in stages of growth of the same species. One might expect the two streams to come into equilibrium in respect to tensions of their blood gasses, the umbilical vein blood returning to the fetus as unsaturated as the venous blood returning to the mother. Were this true, blood received by the fetus should become progressively less saturated with oxygen as gestation proceeds and the full-term fetus would be in imminent danger of

[1] Lecture delivered April 19, 1945.

asphyxiation, which is never the case. It is prevented partly by an anatomical arrangement of the fetal and maternal capillaries within the placenta. Mossman (2), studying the rabbit, found that the maternal and fetal blood streams actually flow in opposite directions. Consequently the fetal blood has the opportunity to become equilibrated with that at the arterial end of the maternal capillary bed. Spanner's (3) investigations reveal a functionally similar mechanism in the human placenta.

A progressive increase in number of hemoglobin-containing blood cells keeps pace with growing need for a fetal oxygen transport mechanism (4). The number of red corpuscles in the human blood at the end of gestation has been found to average 4.5 million per cubic millimeter (5). The amount of hemoglobin is relatively great, reaching about 16 grams per 100 cubic centimeters at the end of gestation. The fetal red corpuscles are large, the average diameter at three months being more than 9 μ and, at birth, approximately 8 μ. The increase in number of corpuscles during development more than offsets decrease in corpuscular size, and hematocrit value and total amount of hemoglobin rise as gestation proceeds toward term.

A fetal type of hemoglobin with enhanced avidity for oxygen at the low tensions prevailing in the placenta has been described in the goat (6), sheep and some other animals (7). A similar avidity has been demonstrated in the whole blood, notably of calves near term (8). The shape and position of the fetal oxygen dissociation curve of whole blood in relation to that of its mother suggests a true chemical difference.

Hemoglobin of cat and human fetuses differs from that of some other species; the fetal and maternal oxygen dissociation curves for hemoglobin solutions are similar in shape and position (9). Nor is there a real difference in the shape of the curves for whole blood (9). And this is in striking contrast to results obtained in such animals as the cow (8) or goat (10). It seems clear that we are dealing with different conditions in the one group from those in the other. The cow and goat are animals with adeciduate placentas. A heavy tissue barrier exists between the maternal

and fetal blood streams. Perhaps a special type of hemoglobin facilitating oxygen transportation across such a barrier has evolved. In the cat and man, the contact between the two blood streams is a closer one, more readily permitting oxygen transfer.

The oxygen-carrying capacity of the blood in late fetal life of man is approximately 21.5 volumes per cent in contrast to 15.5 volumes per cent for the mother's blood at term (9). The actual percentage saturation of fetal blood has been very difficult to determine, for technical reasons. The closest approach to it appears to have been made by Barcroft, Kennedy and Mason (11) who devised a technique for obtaining blood from the umbilical vein or artery without delivering the fetus from the uterus of the sheep and with a minimum of manipulation. They found the blood going to the fetus to be more than 90 per cent saturated with oxygen in half the experiments and not less than 70 per cent saturated in the others. The highest values were found between the 75th and 100th days of gestation (gestation is about 140 days). During the final week lower values were obtained. Other investigators have occasionally encountered high values. In the human fetus for example Bidone (12) reported data which indicate a rather high degree of saturation (approximately 84 per cent) in one experiment.

There seems to be little doubt that a progressive decline in the saturation of blood reaching the fetus takes place in the latter part of gestation. Nevertheless, if we evaluate experimental work critically, we are bound to reach the conclusion that blood supplying the fetus is more nearly saturated than was formerly believed.

A certain amount of dilution of this oxygen-laden blood occurs when it reaches the fetus. Consequently the tissues of the fetus are supplied by less saturated blood than leaves the placenta. Nevertheless, the carotid artery blood of sheep fetuses at caesarean section was found to be as much as 88 per cent saturated with oxygen prior to the last week of gestation (13).

There is some evidence, especially in the latter part of gestation, that the respiratory conditions of the fetus fluctuate. For example, the prelabor contractions of the uterus appear to vary

the degree of saturation of the blood leaving the placenta. Although 95 per cent saturation with oxygen may be attained at times, it is doubtful if the average available to the fetus during the latter part of fetal life is nearly so great, and certainly it is less than it was at an earlier stage of gestation (14).

Throughout prenatal life the need for oxygen is low as compared with that after birth. Oxygen consumption in the latter part of gestation of the sheep fetus has been reported by Barcroft, Kennedy and Mason (15) to be 0.0043 cc./g./min. So long as normal healthy conditions prevail in the placenta a reservoir of oxygen in combination with hemoglobin is constantly present to provide for needs of the fetus. One can calculate that this reservoir amounts to at least 40 cc. of oxygen in the human fetus at term. A factor of safety exists, but is it adequate for meeting grave emergencies?

Occlusion of the umbilical vessels results in a rapid loss of oxygen from the fetal hemoglobin. Even though the fetal heart slows, conserving the store of oxygen somewhat, the amount available in the blood is soon depleted. Nevertheless, the fetal survival time is prolonged considerably beyond the point at which the blood oxygen is used up. There appears to be some other mechanism assuring survival of the newborn after major crises in the fetal respiratory mechanism during birth. Himwich and his colleagues (16) have demonstrated that the survival time for young rats during complete anoxia can be reduced from 50 minutes to three minutes, which is the adult survival time, by preventing utilization of the blood sugar.

No discussion of conditions of respiration in the fetus would be complete without mentioning the course taken by the blood through the fetal heart. The first account of the fetal circulation was given by William Harvey (17), in his treatise of 1628. Moreover, his conception of it was surprisingly close to the truth as we know it today. Only his conviction that there is no pulmonary circulation in the fetus was in serious error. From Harvey's day until the present, the subject of the fetal circulation has provoked lively discussion and controversy. Only recently have

physiological experiments at Northwestern (18), and especially at the Nuffield Institute for Medical Research at Oxford (19), clearly demonstrated the true state of affairs.

The well-oxygenated blood from the placenta reaches the fetus through the umbilical vein and much of it is shunted past the liver by the ductus venosus to reach the inferior vena cava where it is joined by venous blood returning from the lower parts of the fetal body. A small fraction of the inferior caval stream enters the right atrium of the heart; but by far the greater portion passes directly to the left side. The major branching of the inferior caval stream, the *via sinistra* of Barclay, Franklin and Prichard (19), enters the left atrium through the aperture known as the foramen ovale. From the left atrium, blood passes to the left ventricle; thence by the arch of the aorta it is distributed to heart muscle, brain and upper extremities, some passing on into the descending aorta.

Blood returning from the upper parts of the body enters the right atrium of the heart by the superior vena cava. No part of this blood is shunted into the left atrium. All of it passes to the right ventricle, thence by way of the pulmonary trunk to the pulmonary arteries and the ductus arteriosus. A large and rapid circulation through the lungs exists before birth (19, 20). The blood which traverses the ductus arteriosus is carried into the descending aorta to be sent to the lower parts of the body and returned to the placenta by the umbilical arteries.

The significance of this special circulation in the fetus is fairly clear. Certain important organs, the heart, which is the only one working as hard before birth as it will afterward, and the brain, which has to do with activating or inhibiting other bodily activities of the fetus, are kept supplied by reasonably well-oxygenated blood. Here, then, is another mechanism assuring that in the normal course of gestation there is no danger of asphyxiation.

The fetus is capable of performing respiratory movements at an early age. The diaphragm and other respiratory muscles as well as the phrenic nerves are developed long before such movements can be induced. The part of the somatic motor system

which is to be concerned with breathing later on, has its genesis early in embryonic life. Just when the respiratory center begins to form cannot be said. But after the twelfth week of gestation in man (21) or the fifth week in the cat (22) appropriate stimulation, by afferent nervous discharge into the fetal respiratory center, by carbon dioxide acting upon it directly, or by anoxial enhancement of its excitability, results in premature respiratory activity. However, it is doubtful if this newly formed respiratory mechanism actually functions during the normal course of intrauterine existence. The respiratory mechanism of the fetus appears to be a dormant system charged with potentialities long in advance of the time it can be of any use to the fetus.

Premature birth finds the new individual well prepared to breathe. Time does not permit a discussion of all the arguments for and against the occurrence of intrauterine respiratory movements as normal phenomena. On rare occasions rhythmical fetal movements are observed through the mother's abdomen late in gestation (23); but there is no reason to believe that they result in aspiration of amniotic fluid and certainly no evidence in favor of the claim that intrauterine aspiration serves a useful purpose in the developing lung alveoli (24). In the experimental laboratory it is very easy to bring about fetal respiratory movements. In fact, it is difficult to avoid them under the usual laboratory conditions of anesthesia and surgical operations. The fetus, apneic in utero, responds to the same stimuli which cause acceleration of respiration in the adult. Thus, when the pregnant cat is forced to breathe an atmosphere with high carbon dioxide content, respiratory movements of the fetuses can readily be observed (22). But under normal conditions it seems that the fetal respiratory center is insensitive to the level of carbon dioxide prevailing in the fetal blood stream. When the cat breathes atmospheres with low oxygen content and when the saturation of the blood reaching the fetus declines to 40 or 50 per cent, fetal respiratory movements can be observed (25). It would seem that such conditions do not normally prevail.

In experiments with guinea pigs and cats near the end of ges-

tation two types of fetal respiratory movements have been seen. During mild anoxemia and after disturbance of normal uterine tonus, rapid rhythms of shallow movements are encountered. These occur with little increase in fetal skeletal muscle tonus, which is decidedly low in utero. They are weak movements. After prolonged interference with the placental exchange mechanism and during profound anoxia, another pattern of respiratory movement appears. The shallow rhythms cease and after considerable squirming and kicking the fetus begins to gasp rhythmically at a slow rate. The gasps are accompanied by great increase in tonus and the fetal chest expands strongly.

Aspiration of the amniotic contents has been encountered in our experimental animals only under asphyxial conditions. We have been able to obtain no satisfactory evidence that respiratory movements occur normally in utero and certainly no evidence that aspiration of amniotic fluid is a normal phenomenon. Some experiments in guinea pigs will serve to illustrate how intrauterine aspiration can be induced (26).

During the last week or two of gestation we introduced small amounts (0.4 cc. to 1 cc.) of radio-opaque material into the amniotic cavity without using an anesthetic. In 27 fetuses so treated, a large series X-ray films, exposed after various intervals up to 14 days, failed to show lung shadows which would signify that the fetus had aspirated this material. Twenty-five additional experiments were performed. X-rays taken after injection of the radio-opaque material likewise showed no aspiration by the fetuses. The pregnant guinea pigs were then forced to breathe atmospheres low in oxygen or high in carbon dioxide. Fetal respiratory movements could be observed in a number of these specimens. X-ray films were subsequently exposed and in 14 animals showing shallow, questionable or no respiratory movement only two films revealed the radio-opaque material in the fetal respiratory tract. In the remaining 12 experiments, all showing strong respiratory movements, seven positive results were obtained. In addition three other fetuses dying in utero at or about the time of labor or otherwise asphyxiated at birth showed lungs

well filled with the radio-opaque material in consequence of aspiration of the amniotic contents.

You will note that the lungs of the fetuses which executed rapid rhythms of movements, but did not die of asphyxia, were incompletely filled with the radio-opaque material; only the major branches of the respiratory tract contained it. However, those fetuses which died asphyxial deaths showed this substance throughout the entire lung; it was in the alveoli as well as the bronchioles. Intrauterine respiratory movements of fetal guinea pigs do not result in expansion and filling of the lungs with amniotic fluid unless the movements are in the nature of deep gasps.

We have studied the structure of the fetal lung with this point in mind (27). When fetuses were delivered in such a way that respiratory efforts were prevented, histological study of their lungs revealed a true fetal atelectasis. The lung presented a compact appearance; bronchi and bronchioles had definite lumens but the more distal passages did not.

In other experiments the fetuses were delivered only after they had been observed to gasp during asphyxia. Lungs of these specimens resembled those of human still-born fetuses. The alveoli had been opened by the aspiration of amniotic fluid.

Other guinea pigs were allowed to be born normally and to breathe air for various periods of time. The lungs of these animals were only partially expanded after five minutes of air breathing. The unexpanded parts were compact, resembling the truly atelectatic fetal lung.

Seldom is the opportunity provided to observe a state of initial atelectasis in man. Farber and Wilson (28) pointed out that still-born lungs show regions here and there where unexpanded alveoli are visible. Zettelman (29) has studied the lungs of three anencephalic still-born infants and found a state of initial atelectasis. He suggested that a respiratory center either did not exist or was too poorly organized to function at the time they died in utero after eight months or more of gestation.

These experiments demonstrate that the fetal lung is essentially a gland-like organ. In the atelectatic state the alveoli are lined

with cuboidal epithelium. Moreover, the lung appears to function like a gland in fetal life. It would seem that there is an outflow of transudate fluid from the alveoli through the respiratory tree to the amniotic sac, and which normally sweeps the fetal respiratory passages. Potter and Bohlender (30) and Caldwell (31) have studied isolated lobes of fetal lungs which failed developmentally to connect with the exterior by means of the bronchial tree. Caldwell's specimen was particularly striking. It consisted of a mass of alveoli and respiratory bronchioles. The remarkable thing about these specimens is that the alveoli were widely dilated, fluid-filled spaces, for there was no way for the transudate fluid to escape.

Much has been written concerning the conditions under which the infant fails to begin to breathe at birth. Literature is extensive on asphyxia neonatorum, methods of resuscitation, "birth injuries" and relation of these conditions to obstetrical anesthesia; but little experimental work has been done. One need not look far to be aware that asphyxia in late fetal life and at birth is the cause of death in an alarming number of instances. Not only do thousands of infants die each year at birth because of asphyxiation; many others are deeply asphyxiated and heavily narcotized, though they survive. There are indications that some suffer permanent damage to the central nervous system as the result of asphyxia. Some congenital spastics are in the class. Other infants may receive less severe injury of the nervous system, and they constitute an even more exciting problem. Do such infants, suffering asphyxia at birth but escaping clearly defined symptoms of brain damage, become children and adults with neural mechanisms fully equal to those of individuals born normally? Or are some of them to become children of poor learning ability—dullards if not actually mental defectives?

The fetus and newborn are more resistant to deficiencies in their oxygen supply than the adult, but they are occasionally subjected to very severe anoxial conditions at the time of labor or during birth and it should be of great interest to know to what extent the newborn nervous system may be damaged. The im-

probability of obtaining controlled observations in the human species led us to substitute experiments in animals; for this purpose we chose to use the guinea pig (32, 33).

We avoided using a general anesthetic. After local infiltration of the abdomen with procaine solution, one full-term fetus of each litter was delivered promptly to serve as a control. Then the vessels of the uterus were clamped to induce asphyxiation in the remaining fetuses. The duration of asphyxiation varied from four and one-half to 21 minutes. Resuscitation was effected in from a few minutes to an hour and one-half by rhythmical inflation of the newborn lungs with oxygen from a little rubber bag to which a hypodermic needle was attached. This needle was inserted into the trachea. The experiments are illustrated in a motion picture which may be summarized briefly.

Marked changes were produced in the central nervous system of the newborn guinea pig by asphyxiation. A relationship between duration of asphyxia and severity of brain damage could be observed but was not as precise as expected. All animals deprived of a source of oxygen for eight minutes or more showed definite to marked pathology. Those from experiments in which the vessels were occluded only until intrauterine respiratory movements became weak, required little or no resuscitation and thereafter usually showed only slight or indefinite pathology. It is impossible to declare that asphyxia of short duration will cause no nervous system changes or that more prolonged asphyxia will be certain to leave significant permanent defects.

Neurological symptoms were present at birth in all of the animals studied after asphyxiation, regardless of how brief it had been. These included decerebrate states, tremors, convulsive movements, spasticities, paralyses, ataxias and impairment of sensory functions. The motor defects usually failed to persist throughout life and were often only transient. Sensory defects were more permanent. Animals exhibiting the most marked and persistent neurological symptoms also showed the most severe structural changes in the brain.

A closely graded series of specimens from an hour after

asphyxiation to several months afterwards was available for histological study. Multiple capillary hemorrhages and occasionally larger hemorrhages were found in all animals between three hours and five days after resuscitation, in half the animals between 30 minutes and two hours, but in none of those killed by the asphyxia. Great variation in the amount of hemorrhage and in its distribution was encountered and it was quite evident that hemorrhage was not the primary cause of extensive neuron damage.

There was histological evidence of brain swelling in most of the specimens examined between eight hours and four days after resuscitation. Proliferation of neuroglia was observed.

Neurocytological changes were seen in all specimens from one and one-half hours to 21 days after resuscitation. Clouding of Nissl-granule patterns, swelling and loss of stainability with thionin took place in a matter of two to five hours; typical chromatolysis appeared within one or two days. Complete destruction of neurons seemed to be effected as early as four days, though many chromatolysed cells undoubtedly recovered.

Some of the specimens showed generalized changes throughout most of the brain. Others were affected to a marked degree in rather circumscribed regions only. The cerebellum and corpus striatum were not markedly affected. However, the thalamus, cerebral cortex, tegmentum and spinal cord were often severely damaged. The lateral nuclei of the thalamus and the medial and lateral genicular bodies were more frequently involved in destructive processes after asphyxiation than any other regions in the nervous system.

Generalized or regional atrophy followed loss of neurons in two-thirds of the severely asphyxiated and one-half of the mildly asphyxiated guinea pigs' brains. As late as 13 weeks after resuscitation, neurons of the experimental animals stained more darkly and were more shrunken than those of the litter mate controls. Areas of partial necrosis were seen in eight animals. Loss of myelinated nerve fibers appeared to be secondary to cell loss.

It was hoped that before this time a study of the behavioral changes after asphyxiation at birth could have been completed. This study was interrupted more than a year ago and has only recently been resumed. Animals which were asphyxiated at birth together with their litter mate controls were tested in a maze to determine ability to learn a simple alternation problem. When these tests were completed the animals were sacrificed and the brains studied histologically. The brains of 38 animals and their controls, all of which had been subjected to the alternation problem test, have been studied. Twenty-five showed definite structural changes. Of these, 21 were inferior to their controls in the maze test. Some could not learn the simple problem at all. Others quickly forgot the solution. None was superior to its normal control.

It is possible to see similarities between our experiments in guinea pigs and the conditions following asphyxiation in human infants, but much work remains to be done before all questions are answered. Disturbance of the intrauterine respiratory mechanism in man is basically similar to that produced in the guinea pig. It is reasonable to suppose that the same kind of physiological and pathological changes occur in human beings surviving asphyxia and resuscitation at birth as have been demonstrated in experimental animals. Indeed, similar changes have been observed and reported (34). Like our experimental animals, by no means all asphyxiated human infants have permanent manifestations of nervous disease. However, the possibility of inferior mentality, diminished learning ability or simply mental dullness as sequelae of asphyxia at birth should not be dismissed lightly.

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ULTRASTRUCTURE AND THE PROBLEM OF CELLULAR ORGANIZATION[1,2]

FRANCIS O. SCHMITT

SINCE the beginning of microscopy biologists have attempted to find in the structure of cells and tissues some explanation of the mechanism of the complex phenomena manifested by living organisms. The degree of success which attended these efforts was limited by the state of development of the more fundamental sciences of physics and chemistry which furnished the theoretical and experimental tools with which to study the problem.

While most of our knowledge of protoplasmic structure is based on observations with the light microscope either on fresh material or on fixed and stained preparations, the conviction has been growing for almost a century that the structural aspect of protoplasmic organization which is of greatest physiological significance is that which is well below the resolution of the microscope. This view has, in the past, been based largely on speculation; hypothetical giant molecules, for which each succeeding generation of biologists contrived new names, were considered responsible for particular vital phenomena.

But there were also fragments of factual information which confirmed the importance of a molecular organization in protoplasm. Almost a century ago it was discovered that certain cellular constituents show double refraction, from which it was surmised that these constituents possess some sort of quasi-crystalline organization. An important advance came with the application of the Wiener theory of form birefringence to biological studies. This led to the conclusions that cell and tissue fibers are composed of rodlets or fibrils having one dimension small with respect to the wave length of light, and that these submicroscopic fibrils may themselves have a more or less organ-

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ized, micellar structure. In the case of membranes it was more recently shown that the long axes of the submicroscopic units lie in planes parallel with the surface of the membrane.

Meanwhile, ultraviolet microscopy had increased somewhat the resolution obtainable and, in the hands of Caspersson, furnished important clues about chemical constitution. Observations in the dark-field microscope revealed the presence of submicroscopic fibrils and particles of various kinds in intact and fragmented cells and tissues.

Through the pioneering work of Herzog, Meyer and others, the x-ray diffraction technique was applied to biological problems. This early work was concerned chiefly with demonstrating the presence of oriented polypeptide chains in fibrous tissues. Astbury galvanized the interest of biologists by his demonstration of the alpha, beta, and supercontracted states and by his classification of fibers into two general types. The x-ray method cannot as yet be applied to study microscopic structures within individual cells but is applicable to tissues like muscle, tendon and nerve in which there is a sufficient bulk of oriented material to give coherent diffractions. However, the body of knowledge obtained from these fibrous tissues and from extracted proteins may offer suggestions regarding the case of intracellular protein structures.

Stoichiometric considerations of the amino acid residues in protein molecules, which led to Bergmann's theory of the linear sequence of residues in polypeptide chains and to Astbury's (1) attempts to form a unified theory of protein structure, while doubtless premature, furnished biologists with much material for speculation about the nature of the gene, antibodies and other protein structures.

Most recently has come the electron microscope with which "submicroscopic" objects can be visualized directly. With this technique, it may appear, there is no need for difficult physical and mathematical interpretations of the structural implications of the data, as is the case with the indirect methods of polarized light and x-ray diffraction. Now any specimen which can be

gotten thin enough to be penetrable by the electron beam is fair game for the new morphologists! But how may this instrument be most fruitfully employed in furthering our knowledge of the ultrastructural organization of protoplasm? What fundamental problems lend themselves to investigation by the electron microscope and what results have been obtained thus far?

In this paper we shall be concerned chiefly with cells and tissues, omitting the interesting discoveries that have been made with viruses and bacteriophages. It happens that most of the pertinent electron microscope work thus far has been on animal cells hence we shall confine our attention chiefly to such material.

SOME TECHNICAL CONSIDERATIONS

With optically favorable specimens, such as silver particles, the limit of resolution, under standard conditions of operation of present day electron microscopes, is about 50 Å, or somewhat smaller (2). Proteins and other cellular material contain chiefly elements of low atomic number, hence the contrast is poor and the resolving power with such objects is of the order of 50 to 100 Å if the object is suitably thin.

Since specimens to be examined with the electron microscope must be exposed to a high vacuum, cellular material must, in general, be treated with histological fixatives or be frozen-dried. This introduces all of the indeterminacies and artifact formation which plagued the histologists. It represents one of the chief hazards of biological electron microscopy. At present no satisfactory method has been devised to examine material in an aqueous environment although attempts have been made to do so. The energy absorbed from the electron beam is so high in such systems that specimens are thrown into violent thermal agitation, preventing satisfactory microscopy.

Most commercially available instruments employ accelerating potentials of 30 to 60 kilovolts. As a result, biological objects are opaque to the beam if they are thicker than about 0.1 μ (1000 Å). If high resolution is to be obtained the thickness may have to be considerably less. Several methods of sectioning fixed and

embedded material to this order of thickness have been described. All encounter the difficulty of artificial displacement of structures by the blade and some loss of the smaller constituents which may not be well supported throughout such thin sections. The technique permits surveying the general structure of fixed cells but has serious limitations if the finest details are to be observed.

It is difficult to determine the thickness and surface contour of very thin objects, like fibrils or membranes, which have been dried flat on the supporting film. A method very recently described by Williams and Wyckoff (3) appears promising for this purpose. Chromium or other metal is evaporated at an acute angle onto the specimen on its supporting film. Accordingly, more chromium is deposited on one side of objects on the film than on the other and a shadow picture results, from which it is possible to estimate the thickness and surface contour of the object. This method may succeed where stereoscopic methods fail, i.e., with very small objects of low electron-scattering power.

Another method which has hardly been applied as yet in biology is that of surface replicas. Gerould (4) used Heidenreich's silica method to produce replicas of etched bone and tooth surfaces. Electron micrographs of the replicas show great detail of structure especially when viewed stereoscopically. It seems probable that, with suitable modifications, the replica method will be applicable to many biological problems.

A valuable chemical technique is that of electron staining. An electron stain is the equivalent in electron microscopy of a dye in light microscopy and may be defined as any substance which combines preferentially with the object and which contains atoms of high atomic mass to increase the contrast. The application of electron staining techniques to protein fibers will be mentioned below.

OBSERVATIONS ON INTACT CELLS

Cells grown in tissue culture frequently flatten out considerably against the cover glass so that the peripheral portions of

the cell may be very thin. Porter, Claude and Fullam (5) devised methods of depositing such flattened-out cells on specimen holders and examining them in the electron microscope. Structural details were observed in chick embryo cells prepared in this manner which have not been observed by other methods. This technique offers considerable promise if suitable cellular material is chosen and if the complications introduced by fixatives are duly recognized.

OBSERVATIONS ON ISOLATED PROTOPLASMIC CONSTITUENTS

A fruitful way of studying cellular components is to isolate them from cells or tissues by means of teasing, fragmentation, differential centrifugation or chemical extraction. Methods have now been described for the isolation of nuclei, chromosomes, mitochondria, muscle fibrils, glycogen particles and other cellular structures. Few careful electron microscope studies have yet been made on such isolated constituents. Results obtained on such partial systems may not be typical of the structures as they exist in normal cells. But in some cases it may be possible, once the fine structure of a particular constituent has been demonstrated in isolated preparations, to show that the same structure exists in the intact cells or tissues. This was accomplished in the case of certain muscle fibrils by small angle x-ray study of intact muscles (6).

Claude and Fullam (7) have examined mitochondria isolated from a lymphosarcoma of the rat. The mitochondria are opaque to the electrons as prepared but, after chemical extraction, internal structures can be made out. It was found in this laboratory (unpublished) that the mitochondria in the tails of rabbit and pigeon sperm lend themselves to electron microscope examination. The sickle-shaped mitochondria readily separate from their normal packing about the longitudinal fibrils of the sperm tails and are thin enough to show internal structure.

THE FIBROUS PROTEINS

The voluminous cytological literature attests the structural significance of fibers in protoplasmic organization. Not only are

many types of fibrous constituents to be observed in living cells and tissues in the light microscope but there is much evidence, physical, chemical and optical, that the characteristic physical properties of the hyaloplasmic ground substance are due to the presence of submicroscopic rodlets or fibrils. Evidence, mostly indirect in nature, has been adduced for the existence of a system of intracellular fibrils which has come to be known as the cytoskeleton. Polarized light studies of egg cells during and after centrifugation suggest the existence in these cells of a fibrous organization which can be displaced centrifugally (8, 9). Polarized light and electron microscope studies demonstrate that the axon of the fresh nerve fiber, though appearing structureless in the light microscope, contains submicroscopic fibrils oriented parallel with the fiber axis.

Even membranous structures, like the plasma membrane, may be found to be fibrous. Wolpers' (10) electron microscope studies of the limiting envelope of the erythrocyte suggest that this thin structure may be composed of a felt-work of extremely thin but quite long protein fibrils.

In view of the important structural role played by fibers, particularly protein and conjugated protein fibers, it is desirable that every effort be made to obtain as much information as possible about their ultrastructure. We shall consider below several fiber types about which some information is at hand. It will be obvious that only a small fraction of the biologically interesting fibers have thus far been studied. Particularly conspicuous is the lack of data on chromosomes and other nucleoprotein fibers. Caspersson's (11) work has contributed important clues about the location of protein and nucleic acid components in chromosomes and some x-ray work has been done on extracted nucleoprotein fibers. But as yet detailed electron microscope investigations are lacking.

It is beyond the scope of this paper to discuss the theory of protein fiber structure at the atomic level. The results obtained from wide-angle x-ray diffraction studies have been summarized in various places recently (1, 12). Rather, we shall be concerned

with the somewhat larger structural aspects which are revealed by electron microscope and small-angle x-ray diffraction studies, dealing chiefly with those which have been carried out in this laboratory by my colleagues, R. S. Bear, C. E. Hall and M. A. Jakus.

General Characteristics of Protein Fibrils

In the electron microscope protein fibers are observed to be composed of an aggregation of very thin, long fibrils (13). They have widths usually in the range from about 100 to 1000 Å and lengths ranging from a few to many microns, possibly even millimeters. Accordingly, the asymmetry ratio may be enormous, of the order of hundreds and possibly thousands.

In some cases, like collagen, the fibrils may be observed to fray longitudinally, indicating that they consist of a parallel packing of still thinner longitudinal components. In other cases no such fraying is observed and it is possible that these represent the "molecules" or macromolecular units studied in ultracentrifuge, diffusion, viscosity, osmotic pressure and streaming double refraction experiments. In this respect they would be intracellular analogs, from the structural standpoint, of the fibrous plant virus proteins.

Because of their thinness it is difficult to determine their shape in cross-section with any degree of accuracy. In some cases, such as certain clam muscle fibrils, they appear to be flat ribbons, having essentially a two-dimensional grid-like structure.

A characteristic which may prove typical of many protein fibers is the presence of a periodic repeating pattern in the axial direction which may have dimensions of the order of some hundreds of Ångstrom units. No such large periods have been found in carbohydrate fibers or silk, in which the number of different constituent chemical building stones is relatively small. Bear (6) points out that proteins like keratin, collagen, and muscle contain many different amino acids in the molecule and this property may be associated with the formation of large axial repeating periods.

Muscle Protein

Electron microscope examination of purified myosin, obtained from mammalian muscles, reveals the presence of fibrils about 100 Å in width and several microns long (14). The relationship of these fibrils to the characteristic cytological appearance of striated muscle has not yet been satisfactorily demonstrated. Published electron micrographs of thin microtome sections of muscle (15, 16) are not satisfactory for the purpose. Nor have detailed data been published as yet on the structure of striated muscle as studied by the small-angle x-ray technique.

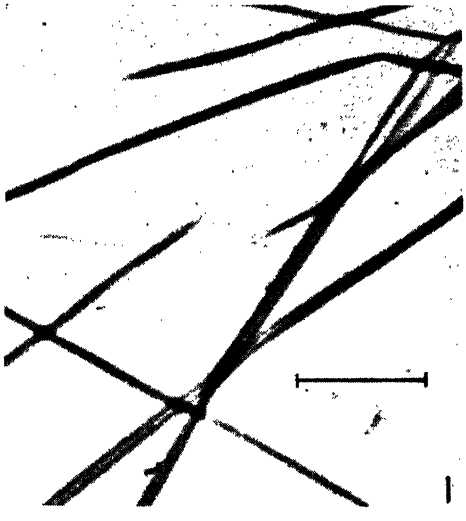
A particularly interesting type of muscle fiber is that found in the adductor muscles of *Venus* and certain other molluscs. Macerated in salt solutions (ca. 0.25 M KCl), these fibers readily separate into long, slender fibrils 500 to 1000 Å in width and several microns long (17). They manifest no regular internal structure in the electron microscope (Fig. 1). However, when treated with phosphotungstic acid (ca. 0.1%) before drying on the specimen holder, the fibrils show a remarkable fine structure consisting of a regular repetition of dark and light bands (Fig. 2). The distance from one dark band to the next is surprisingly constant in individual fibrils and is equal to 145 Å on the average. When the phosphotungstic acid stain is appropriately applied the cross bands show fairly discrete spots (Fig. 3). These spots lie on two sets of diagonals making different angles with the fiber axis. The geometry of the pattern is such that there is a repeating structure along the fiber axis every 720 Å (Fig. 4).

FIG. 1. Fibrils from adductor muscle of *Venus mercenaria,* unstained. ×17,000. Scale indicated on each figure represents 1 μ unless otherwise specified.

FIG. 2. Fibrils from adductor muscle of *Venus mercenaria* stained with phosphotungstic acid to show banded structure. ×70,000. Distance between bands is 145 Å.

FIG. 3. Same as Fig. 2. Appropriate staining reveals discrete spots which lie on diagonals which make different angles (α and β) with the axis. ×145,000.

FIG. 4. Diagrammatic representation of stained lattice in *Venus* muscle fibrils.



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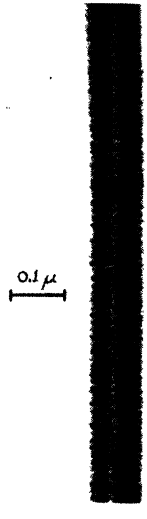

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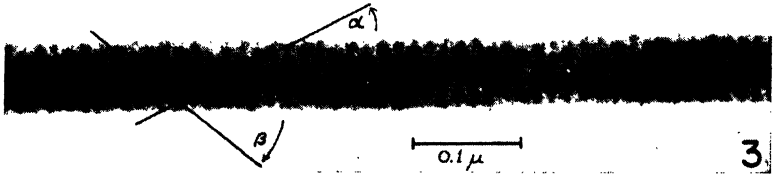

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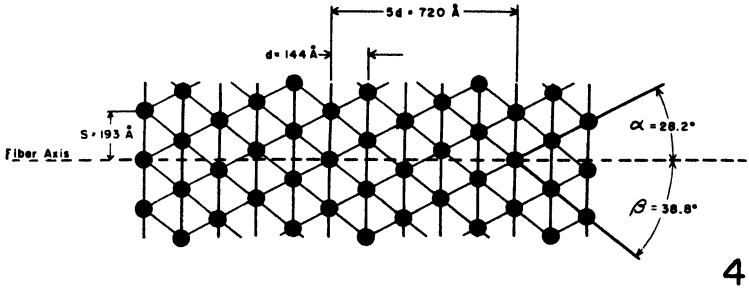

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This structure is in close agreement with small-angle x-ray diffraction data obtained by Bear (6) from the same material and represents the most striking example thus far obtained of direct visualization of the electron density variations in a protein fiber conforming in detail with that deducible from the x-ray analysis.

This geometrical pattern can be observed also in fibrils obtained from muscles which had been fixed in alcohol. It is possible, therefore, to examine fibrils from muscles which had been in a state of extension or contraction at the time of fixation. The pattern was found to be essentially the same in both cases. The interpretation of this invariance of structure of the fibrils in terms of the contractile process in these muscles is not yet clear.

Collagen

Collagen fibrils also show an axially banded appearance in the electron microscope (18), the period (640 Å) agreeing closely with that found by x-ray diffraction studies (19). No staining is required to demonstrate this periodic structure; one has only to tease the fibrils from a bit of tendon and examine them directly in the electron microscope (Fig. 5). This structure is characteristic of all collagen fibers thus far examined and an experienced observer can readily distinguish collagen from other fibers in cell or tissue preparations.

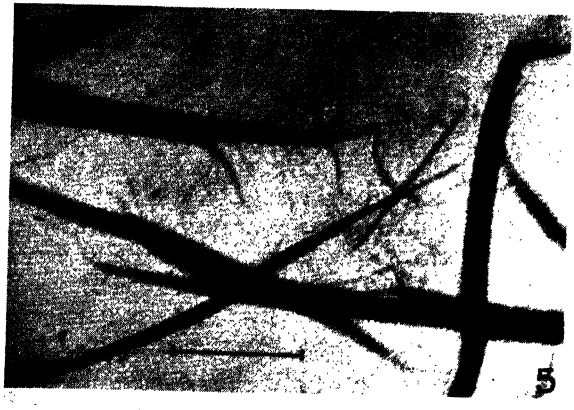
When collagen fibrils were stained with phosphotungstic acid the electron micrographs revealed not merely one dark and one light band per period but a series of bands, depending on the degree of extension of the fibril (Fig. 6). Apparently a number of regions exist within the repeating period which combine preferentially with the stain; extension of the fibril causes certain bands which, in the unextended fibrils appeared to be singlets, to break up into doublets. This structure is qualitatively consistent with that deducible from the small-angle x-ray data.

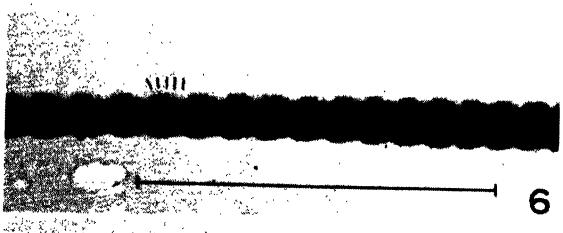
The above experiments with clam muscle and collagen fibrils foreshadow the important role which electron stains may be ex-

pected to play in future investigations of protein ultrastructure. The chemical interpretation of the fine structure revealed in the stained muscle and collagen fibrils is not yet clear though the combination of these proteins with phosphotungstic acid is probably a sort of tanning reaction. Unfortunately, despite years of research devoted by leather chemists to the chemical mechanism of tanning, little is known about the details of the reaction. Silicotungstic, phosphomolybdic and chloroplatinic acids also stain the structures described above, but the resultant contrast is not quite so great as with phosphotungstic acid.

These reagents belong to the class of coordination compounds known as heteropolyacids (20). The molecules may be relatively large, having molecular weights of several thousand. This potentiates their staining ability since each molecule may contain from 6 to 24 or more heavy atoms like tungsten, molybdenum, platinum, and others. The highly olated vegetable tans and chrome complexes used by tanners may also have high particle weight and size. There has been much controversy as to whether such reagents produce tanning by combination with terminal polar groups of side chains, with the peptide linkages or with other groupings in the protein. In all events, these electron stains, being highly water-soluble, doubtless penetrate the protein fibril in aqueous regions and combine with hydrophilic groups located in these regions.

If stains can be devised which will combine differentially with specific chemical groups in the protein, electron micrographs may reveal the location of these groups. To what extent such studies will contribute to our knowledge of the amino acid composition and configuration in fibrous proteins remains to be demonstrated. With the introduction of electron stains we encounter difficulties similar to those which result from the use of fixatives and stains in cytological technique. The reagents may bring out certain structures with great clarity, yet these structures may bear no obvious relation to those existing in the cell under normal conditions. Hence stained structures should be interpreted with great caution. In the cases of clam muscle and





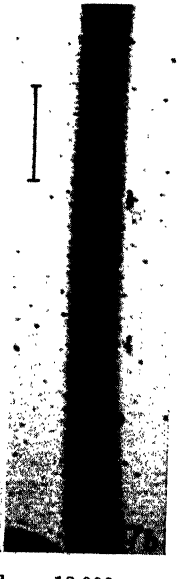


FIG. 5. Collagen fibrils from rat-tail tendon, unstained. ×18,000.

FIG. 6. Collagen fibrils from rat-tail tendon stained with phosphotungstic acid. ×49,000.

FIG. 7. Trichocyst from *Paramecium*. 7a shows dense tip, 7b shows banded shaft. ×14,000.

FIG. 8. Banded fibrils from fibrin clot in spinal fluid obtained from patient with tuberculous meningitis, stained with osmic acid. ×16,000.

collagen fibrils there can be no doubt about the existence of the structure in the normal tissues, for x-ray evidence obtained with intact tissue is in good agreement with that revealed by the stains in extracted and dried fibrils.

Fibrin

Students of protoplasmic structure are interested in the fibrinogen-fibrin system because of clues which it may give to the processes by which fibrous structure may be formed in protoplasm. While the mechanism of the formation of fibrin by the interaction of fibrinogen and thrombin is still poorly understood, data have been obtained recently bearing on the shapes and internal structure of the fibrinogen and fibrin molecules. Fibrinogen is a very asymmetric molecule with a particle weight of the order of 500,000 and a length estimated to be of the order of 900 Å (21, 22). Fibrin differs little from fibrinogen in chemical composition and presumably consists of fibrinogen molecules bonded together in an undetermined fashion by the action of thrombin.

From wide angle x-ray diffraction studies, Bailey, Astbury and Rudall (23) conclude that both fibrinogen and fibrin belong in the keratin-myosin class of protein fibers, since they give characteristic, though somewhat imperfect, alpha and beta patterns.

Some electron microscope studies have been made of the process of blood clotting (24). Ruska and Wolpers (25) observed that fibrils, obtained from the pellicle which forms in the spinal fluid in cases of tuberculous meningitis, appear banded in the electron microscope. Hobson, in this laboratory, observed a similar structure in one case out of seven examined (see Fig. 8). Axial periodicity has not yet been demonstrated in fibrin formed from highly purified fibrinogen and thrombin. Ruska and Wolpers believe the production of periodic patterns requires a very low reaction velocity to permit the fibrinogen molecules to aggregate in this quasi-crystalline fashion. The electron microscope observations are important not only for the information they give about the structure of fibrin, but also for clues they furnish about

the mechanism of formation of this protein and possibly about the structure of fibrinogen itself.

Neurofibrils

Axoplasm is one of the most difficult systems to analyze structurally because of the great chemical lability of its highly hydrated components. Polarized light studies (26) indicated the presence, in the normal nerve axon, of submicroscopic fibrils oriented parallel with the axis. These very thin fibrils presumably constitute the lattice on which materials deposit to form the neurofibrils visible in the light microscope. The system is too tenuous and hydrated to give useful x-ray diffractions. Richards, Steinbach and Anderson (27) observed a tortuous system of thin fibrils in specimens of axoplasm, extruded from giant fibers of the squid, which they relate to those inferred from the polarized light studies. Special methods will have to be devised to permit electron microscope investigation of the fine structure of these protein fibrils and their chemical relationship with the surrounding axoplasm.

Trichocyst Sheath Protein

Certain protozoa possess structures, called trichocysts, embedded below their surface membrane which may be extruded explosively in the form of elongated fibrils when the animal is attacked or stimulated electrically. In the process the trichocyst elongates six to ten-fold, due to a rapid process of swelling. The extruded trichocysts, viewed with the electron microscope, are seen to possess a dense pointed tip (Fig. 7a) and an elongated shaft which is very regularly cross-striated (Fig. 7b). Following preliminary observations (13) Jakus made a close study (unpublished) of the phenomenon. She concludes that the striated material represents an extremely thin proteinaceous sheath. This sheath, with its characteristic axial periodicity (ca. 550 Å) is probably preformed and highly folded in the resting trichocyst. On stimulation the material enclosed within the sheath swells enormously and rapidly (order of a few milliseconds), causing the trichocyst to be expelled from the cell like a torpedo.

The sheath protein may be added to the list of those which show large periodic variations in density in the axial direction. Its closer chemical and structural characterization will be of great comparative biochemical interest since it seems to be an extremely primitive fibrous protein.

POLARITY IN PROTEIN FIBERS

Examination of the electron micrographs of stained collagen and clam muscle fibrils (Figs. 4 and 6) shows that these fibrous proteins are not symmetrical but manifest polarity of structure. In somewhat extended collagen fibrils stained with phosphotungstic acid the five dark bands in the repeating period are not equally spaced or equally dense. The fibril might be considered to "point" in one direction or the other, depending on the reference point which is arbitrarily chosen in the banded structure. One may readily recognize whether adjacent fibrils have the same or opposite axial polarity. There is some evidence that collagen fibrils may be laid down over macroscopic regions in certain tissues with all the parallel fibrils having the same polarity.

From Fig. 4 it may be seen that clam muscle structure is also polarized. The two diagonals make different angles with the axis, hence the flat, ribbon-like fibrils would appear differently if rotated through 180° about the long axis.

Since the differences in density of the stained collagen and muscle fibrils reflect differences in chemical composition or configuration, the polarization may imply differential chemical and physiological properties. The structural polarity also has interesting implications in the embryogenesis of fibers which show such polarity.

THE MECHANISM OF FIBROGENESIS

Since protoplasm apparently employs fibrous structures very largely in forming its microcosmic architecture, the process by which these fibrils are formed and recruited into an orderly organization becomes fundamental not only in biology but in medicine as well. Recent work on protein structure is useful, if not to solve the problem, at least to suggest lines along which it may profitably be approached experimentally.

Two possibilities suggest themselves for the formation of fibers: 1) by lateral and longitudinal aggregation of extremely thin preformed fibrous units, possibly the highly elongated native protein molecules themselves; 2) by columnar aggregation of relatively symmetrical soluble globular molecules.

Strongly favoring the first possibility is the structure observed in muscle, fibrin and collagen. The case of collagen is particularly striking and may be mentioned in some detail.

Collagen fibrils fray longitudinally, the finest observable constituent fibrils being close to the resolution of the electron microscope. In dilute acetic acid the collagen fibrils of rat-tail tendon dissolve completely. The streaming double refraction, viscosity, and ultracentrifugal properties of the filtered, water-clear solution show that highly elongated particles are present, although they have not as yet been detected with the electron microscope, presumably because they have at least one dimension smaller than the resolution of the electron microscope. Upon neutralization of the acid solution, fibrils are promptly formed and these fibrils show all the fine structure demonstrated in native collagen fibrils treated with electron stains.

While the process is far from understood, it seems probable that, when the ionic environment is favorable, the elementary fibrous units, called protofibrils by Schmitt, Hall and Jakus (18), aggregate laterally to produce the fibrils observable in the electron microscope. Significant is the fact that these protofibrils aggregate with each other "in phase" with respect to their axial fine structure, giving even quite wide fibrils a cross-striated appearance in the electron microscope.

The weight of evidence now available supports the view that microscopically visible collagen fibers are formed extracellularly in the proximity of fibroblasts. The nature of the fiber-forming material is not yet understood. The theory that fibrin may be chemically transformed into collagen (28) is based chiefly on histological staining evidence which is equivocal (29). Alternatively, it may be supposed that collagen protofibrils or their precursors, produced within the fibroblasts, find their way into

intercellular spaces where the chemical environment is favorable to their aggregation to form typical collagen fibrils. Since collagen fibrils can now be observed and identified in the electron microscope the way is open to a direct experimental approach to the problem.

According to the second hypothesis, fibrils may be formed by a columnar aggregation of soluble globular protein molecules which are not highly asymmetric in shape. The essential feature of this view is that the globular molecules become linked end-to-end without extensive denaturation such as occurs when these proteins are made fibrous by heat denaturation. Waugh (30) has emphasized this possibility and has described experiments with insulin which exemplify the process. By the action of acids and alkalies he demonstrated that insulin molecules may be *reversibly* converted into fibrils which, like certain tissue fibrils, have widths between 100 and 200 Å and are microns long. This fibrous modification can be converted back to soluble insulin molecules without appreciable loss in biological activity or ability to form characteristic insulin crystals. If such processes occur in cells it may be presumed that they do so under the influence of specific enzymes.

The two possible mechanisms of fibrogenesis discussed above are not mutually exclusive nor the only ones which might be suggested. While it is possible at present to offer only speculations about the fundamental processes of structure formation in cells, techniques are now at our disposal which should facilitate their further analysis.

CONCLUSIONS

Protoplasmic structure determination has, in the past, been considered the task of the morphologist. But if his only optical tool is the light microscope he can study only the relatively gross aspects of the structure. This is of little help to the physiologist in explaining the mechanism of cell and tissue function.

Physiological function involves the ordered interaction between the structural "machinery" of the cell and the surrounding energy-furnishing aqueous milieu containing salts, enzymes, hor-

mones and a wide variety of metabolites. We have seen that the really characteristic structure of the machinery—the intracellular reacting system—is to be observed by studying its elementary units. From the investigations of recent years it has become clear that one of these structural units is the protein fibril which may be microns in length but from a few hundred to possibly less than 50 Å in width. These minute fibrils aggregate into parallel or anastomosing bundles to form fibers visible in the light microscope. Or they may be interwoven into very thin fabrics to form membranes, of which the plasma membrane may be an example.

Fortunately a high degree of order exists in the elementary structural units and this makes it possible to obtain valuable data by the use of x-ray diffraction and electron microscope techniques. These techniques have been applied to the problem only fairly recently and much development will be required before the limits of their usefulness can be estimated.

The task therefore resolves itself very largely into one of molecular and macromolecular morphology. At this level of organization the boundaries between morphology, physiology, and biochemistry very largely disappear. The investigation of protoplasmic ultrastructure presents a stimulating challenge to physicists, chemists, and biologists alike and is eminently worthy of their best efforts. If representatives of these sciences will join forces in a common assault upon the problem further progress may be expected to be rapid.

The normal function of a cell or tissue involves the integrated action of all of the structural components. This integration constitutes a much higher level of organization than that which we have been discussing, one with which the biologist feels more at home. It is an unfortunate though understandable characteristic of those who focus their attention on the lower levels of structure to have a myopic view of the cell or tissue system as a whole. However, this is perhaps less regrettable than the hyperopia which characterizes many of those who are content to restrict themselves to purely descriptive studies of structure for

its own sake, usually at the level of light microscope resolution or somewhat lower. Until we have progressed sufficiently with the difficult problems of molecular morphology it is unlikely that rapid advance will be made with those which involve far higher levels of organization.

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